FORAGING ECOLOGY AND NUTRITIONAL STRESS OF TUFTED PUFFINS (*FRATERCULA CIRRHATA*) INFERRED FROM STABLE ISOTOPES, FATTY ACID SIGNATURES, AND FIELD ENDOCRINOLOGY

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A

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Abstract

Prey availability has a major impact on the reproductive output of seabirds, yet information on seabird diets throughout the breeding season is often lacking. Although reduced prey availability is known to affect the growth and survival of nestling seabirds, few studies have demonstrated similar effects on indices of adult body condition. I used stable isotopes and fatty acid (FA) signatures to investigate seasonal and age-related variation in the foraging niches of tufted puffins (*Fratercula cirrhata*). I conducted captive feeding experiments to determine whether inferences based on these techniques are affected by moderate food restriction during growth. I also examined how adult puffins prioritize the competing goals of maximizing the growth rate of their offspring and maintaining their own condition, as measured by body mass and by the stress hormone, corticosterone (CORT). Food restriction during nestling growth affected adipose tissue FA signatures and resulted in blood that was depleted in ^{15}N and ^{13}C relative to well-fed controls. However, effects of nutritional restriction on $\delta^{15}N$, $\delta^{13}C$, and FA signatures were small compared to variability in prey, indicating physiological effects do not preclude use of these techniques as dietary tracers. Stable isotopes and FA signatures of free-living adults indicated foraging niches changed over the course of the breeding season. Stable isotopes suggest chick-rearing adults and nestlings feed at the same trophic level while FA signatures indicate that parents feed nestlings a diet different from their own. Body mass of adult puffins declined between incubation and chick rearing periods. For females the magnitude of mass decline did not differ between years, whereas for males the decline was greater in the year where young puffins fledged at a lower mass. In a separate analysis, baseline CORT values of adults of both sexes did not differ between years, but were lower than those observed in a separate study area during two consecutive years with low rates of nestling growth and survival. Assuming elevated CORT and reduced body mass impact survival and/or future fecundity, these results suggest the cost of reproduction may be higher for those adults able to fledge young in years characterized by low productivity.

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General Introduction

The reproductive output of seabirds reflects prey availability which, in turn, is affected by climate-driven changes in physical forcing that occur at a variety of spatial and temporal scales (Anderson & Piatt 1999, Benson & Trites 2002, Mann & Lazier 2006). Understanding how changes in food web structure affect the productivity and survival of seabirds requires information on diets throughout the year. Although stomach content analysis is the most common method used to investigate seabird diets, it is subject to well-known bias associated with the rapid digestion of soft-bodied prey and the retention of hard-parts (e.g. Votier et al. 2003). Therefore, I used stable isotopes and fatty acid signatures to investigate annual, seasonal and age-related variation in the foraging niches of tufted puffins (*Fratercula cirrhata*), a pelagic diving seabird. Although analyses of stable isotopes and fatty acid signatures are potentially useful to address these issues, these methods may be affected by changes in physiological condition (Hobson et al. 1993, Gannes et al. 1998). Therefore, I also examined whether estimation of trophic level of feeding, foraging location, and/or dietary shifts based on stable isotopes and fatty acid signatures can be compromised due to the effects of nutritional state on physiological processes.

The response of animals to changes in food availability and/or quality is also determined by their life-history strategy (Lack 1968). As long-lived, iteroparous species, seabirds are generally expected to favor survival over reproduction during years of low food availability (Williams 1966). If foraging conditions deteriorate below some threshold level, seabirds may elect not to reproduce, thus avoiding the "cost of reproduction". Breeding seabirds experience a "cost of reproduction" but whether this cost varies with current foraging conditions is not clear (Erikstad et al. 1998, Golet et al. 1998). I investigated how adult puffins prioritize the competing goals of maintaining their own condition and maximizing the growth rate of their offspring. I assume that lower body condition (mass scaled to structural size) and higher levels of the stress hormone corticosterone (CORT) are costly, resulting in reduced survival and/or decreased future fecundity. Experimental elimination of reproductive effort in the black-legged kittiwake

(*Rissa tridactyla*) results in decreased levels of CORT, decreased seasonal deterioration of body condition, and increased winter survival (Golet et al. 2004). This provides some circumstantial support for these assumptions.

In response to changes in prey availability, adult seabirds can adjust their foraging strategies and/or their reproductive effort. In contrast, nest-bound chicks are completely reliant on their parents to meet their nutritional demands. However, nestlings can alter their behavior to signal parents to increase food provisioning. Begging behavior is likely triggered by hormones, such as CORT, and costs associated with endocrine control may play a role in ensuring this behavior is honest (Kitaysky et al. 2001, Loiseau et al. 2008). In the final chapter of this thesis, I examine whether CORT is associated with begging behavior in a species that routinely experiences prolonged nutritional restriction during development.

CHAPTER 1. Information on trophic level of feeding and foraging location provided by analysis of nitrogen and carbon stable isotopes may provide valuable insight into the mechanistic link between ocean climate and reproductive success in seabirds observed at some colonies (e.g. Gjerdrum et al. 2003). However, effects of nutritional status on isotopic fractionation have the potential to produce spurious correlations between measures of ocean climate (e.g. sea surface temperatures) and $\delta^{15}N$ or $\delta^{13}C$ values of predator tissues. Severe chronic food restriction and natural fasting events have been shown to result in body tissues becoming enriched in $\delta^{15}N$ (Hobson et al. 1993, Cherel et al. 2005), although effects of moderate restriction have rarely been tested (Kempster et al. 2007). In chapter 1, I determine the effects of a 50-55% reduction in food intake on $\delta^{15}N$ and $\delta^{13}C$ values in blood cells and whole blood of tufted puffin chicks. I then examine whether effects of food deprivation on isotopic fractionation are sufficient to confound estimates of foraging location and trophic level of feeding derived from isotopic analysis.

CHAPTER 2. Analysis of fatty acid signatures is increasingly being used as a tool to study foraging ecology and food web dynamics in marine ecosystems (reviewed in Budge et al. 2006). Fatty acid signatures are most commonly used to infer spatial or

temporal differences in diet (Iverson et al. 1997, Walton et al. 2000) or to confirm the importance of certain prey types in predator diets (Raclot et al. 1998, Käkelä et al. 2006, Budge et al. 2007). Signatures can also be used to quantitatively estimate predator diets using a statistical model that determines the combination of prey fatty acid signatures that is closest to the predator signature after accounting for effects of metabolism (e.g. the QFASA method: Iverson et al. 2004). Similar to analysis of stable isotopes, fatty acid signatures may provide useful insight into the effects of environmental conditions on seabird diets. However, the effect of nutritional status on fatty acid metabolism in growing seabirds has not previously been addressed. In chapter 2, I examine the effects of a 50% reduction in food intake on adipose tissue fatty acid signatures in tufted puffin nestlings. Because food restriction coincided with a shift in diet, I used a repeated sampling design to determine if observed effects were due to differences in rates of fatty acid turnover or were a consequence of altered fatty acid metabolism.

CHAPTER 3. While seabird productivity can be limited by the capacity of adults to obtain food for their young, breeding failure often occurs during incubation or prior to egg-laying (e.g. Chastel et al. 1995, Wanless et al. 2005) - time-periods for which diets are poorly characterized in most species. Furthermore, according to central-place foraging theory, chick-rearing adults that transport food in their bills should attempt to maximize the rate of energy provided to their offspring by selecting large, high quality prey items to feed their young (Orians & Pearson 1979). When feeding for self-maintenance, adults may increase their rate of energy intake by selecting smaller prey items that occur in highly predictable and dense aggregations. In chapter 3, I use stable isotopes and fatty acid signature analysis to infer age- and stage-dependent foraging niches in free-living tufted puffins breeding on Chiniak Island near Kodiak, AK. I also include an index of body condition in my analysis of stable isotopes to examine whether nutritional status might be affecting estimates of trophic level of feeding based on $\delta^{15}N$.

CHAPTER 4. Reduced prey availability is known to affect rates of nestling growth and survival in seabirds but consequences for breeding adults are unclear. In some species, the cost of reproduction appears to be relatively fixed and consequences of low

food availability are borne primarily by nestlings (e.g. Saether et al. 1993, Weimerskirch et al. 1999, Duriez et al. 2000), whereas in other species, these consequences are shared by adults and nestlings (Weimerskirch et al. 2001, Gaston & Hipfner 2006). Moreover, sex-specific roles in parental care and differences in the capacity to store energy reserves may lead to sex-specific modulation of body condition. In chapter 4, I explore how adult male and female tufted puffins prioritize the competing goals of maintaining their own body condition and maximizing the growth rate of their offspring. Specifically, I examine the seasonal dynamics of adult body condition in tufted puffins breeding on Chiniak Island in 2004-2005 and relate inter-annual differences in body condition to the growth rate and survival of nestlings. I also develop a body condition index (BCI) and examine whether this BCI must be calculated separately for each sex in this species to avoid intra- and inter-sexual bias.

CHAPTER 5. Animals respond to environmental stressors by activating the hypothalamus-pituitary-adrenal (HPA) axis, resulting in increased circulating levels of corticosterone (CORT; Wingfield et al. 1997). Elevated CORT induces the mobilization of stored energy reserves and promotes foraging behavior at the expense of reproduction (reviewed in Sapolsky et al. 2000). Previous studies have shown that in some species of seabirds, baseline and maximum stress-induced levels of CORT are correlated with reproductive success (Kitaysky et al. 1999, Buck et al. 2007, Kitaysky et al. 2007). Seabird productivity is often affected by food availability (e.g. Croxall et al. 1999), and CORT may therefore provide useful information on marine resource availability. However, if CORT is to be used as an effective monitoring tool, it is critical to first determine whether circulating levels are affected by stage of reproduction. Furthermore, measures of total CORT concentration in plasma includes hormone bound to corticosteroid binding globulin (CBG) and unbound or free CORT. It has been suggested that because only free hormone is available for diffusion into tissues, it may be a more reliable measure of physiological stress (Breuner & Orchinik 2002, Love et al. 2004). In chapter 5, I investigate total CORT, CBG, and free CORT in adult tufted puffins during three stages of reproduction: prior to egg laying, late incubation, and late chick-rearing. I

also include body condition index as a covariate in analyses to determine whether CORT and/or CBG vary with energy stores. I then compare baseline and maximum stress-induced levels of total CORT in chick-rearing adults from a high productivity colony to levels in adults from a low productivity colony located in a different study area.

CHAPTER 6. In many species, nestlings also respond to reduced food intake by increasing plasma CORT levels. Short term elevation of CORT may benefit nestlings by promoting mobilization of stored energy reserves and triggering increased begging behavior whereas the costs of chronic elevation of CORT may help to ensure begging is an honest signal of nutritional state (Kitaysky et al. 2001, Loiseau et al. 2008). However, in species where free-living chicks routinely experience extended periods of reduced energy intake, such as tufted puffins, the high costs associated with chronic elevation of CORT may lead to non-responsiveness or suppression of the HPA axis during nutritional restriction (Kitaysky et al. 2005). In chapter 6, I examine the relationships between free CORT, total CORT, nutritional state, and begging behavior in tufted puffin nestlings fed high and low-calorie diets.

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Chapter 1: Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds[1]

1.1 Abstract

When using stable isotopes as dietary tracers it is essential to consider effects of nutritional state on isotopic fractionation. While starvation is known to induce enrichment of ^{15}N in body tissues, effects of moderate food restriction on isotope signatures have rarely been tested. We conducted two experiments to investigate effects of a 50-55% reduction in food intake on $\delta^{15}N$ and $\delta^{13}C$ values in blood cells and whole blood of tufted puffin chicks, a species that exhibits a variety of adaptive responses to nutritional deficits. We found that blood from puffin chicks fed ad libitum became enriched in ^{15}N and ^{13}C compared to food-restricted chicks. Our results show that ^{15}N enrichment is not always associated with food deprivation and argue effects of growth on diet-tissue fractionation of nitrogen stable isotopes ($\Delta^{15}N$) need to be considered in stable isotope studies. The decrease in $\delta^{13}C$ of whole blood and blood cells in restricted birds is likely due to incorporation of carbon from ^{13}C-depleted lipids into proteins. Effects of nutritional restriction on $\delta^{15}N$ and $\delta^{13}C$ values were relatively small in both experiments ($\delta^{15}N$: 0.77 and 0.41‰, $\delta^{13}C$: 0.20 and 0.25‰) compared to the effects of trophic level of feeding and/or foraging location, indicating physiological effects do not preclude use of carbon and nitrogen stable isotopes in studies of seabird ecology. Nevertheless, our results demonstrate that physiological processes affect nitrogen and carbon stable isotopes in growing birds and we caution isotope ecologists to consider these effects to avoid drawing spurious conclusions.

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1.2 Introduction

Analysis of $^{12}C/^{13}C$ and $^{14}N/^{15}N$ stable isotopes in animal tissues provides a useful means of delineating dietary sources, determining trophic level of feeding, and tracking migratory movements (reviewed in Hobson 1999, Kelly 2000). This technique has proved particularly useful to estimate foraging location and trophic level of feeding in seabirds (Hobson et al. 1994, Forero et al. 2004, Cherel et al. 2006) where conventional techniques for diet determination are logistically impractical and biased (Wilson et al. 1985, Votier et al. 2003). However, interpretation of isotopic data in studies of animal ecology is complicated by effects of nutritional status on biochemical pathways and physiological processes (Gannes et al. 1998). Despite widespread awareness of physiological processes that may affect diet-tissue fractionation, and a call for more laboratory experiments made nearly a decade ago (Gannes et al. 1997), few experimental studies have been conducted to assess the effects of nutritional status on fractionation of carbon and nitrogen stable isotopes. The paradigm emerging from studies exposing birds to experimental food deprivation postulates that animal tissues become enriched in ^{15}N following nutritional restriction due to catabolism of endogenous protein stores, recycling of metabolic amino acids, and discrimination against the heavier isotope during formation of nitrogenous wastes (Macko et al. 1986, Hobson et al. 1993, Gannes et al. 1998). Although this effect is well-established in fasting animals that have no access to exogenous nitrogen (Hobson et al. 1993, Oelbermann and Scheu 2002, Cherel et al. 2005a), experimental studies employing moderate food restriction have rarely been undertaken (Kempster et al. 2007).

Seabirds provide their young with prey that varies in its abundance and distribution, often resulting in moderate food restriction during growth when demands for energy and protein are high. Reproductive success and rates of nestling growth of tufted puffins (*Fratercula cirrhata*, hereafter: puffin), for instance, are affected by climate-induced changes in sea surface temperature (Gjerdrum et al. 2003) presumably due to variation in prey availability. Information on trophic level of feeding and foraging

location provided by analysis of stable isotopes may therefore be useful for elucidating the mechanistic link between sea surface temperatures and reproductive success. However, effects of nutritional restriction on nitrogen diet-tissue fractionation, assuming it occurs, may produce a spurious correlation between rates of nestling growth and estimates of trophic level of feeding. Because growth rates of puffin chicks vary widely between colonies and years (Piatt and Kitaysky 2002, Gjerdrum et al. 2003) as well as between individuals within a colony (Williams, unpublished data), we set out to determine the effects of moderate food restriction on carbon and nitrogen stable isotopes in growing puffins.

Nutritional status is a function of nutrient intake versus demand, and the physiological and metabolic compensatory mechanisms that minimize the discrepancy between the two (King and Murphy 1985). We hypothesized that the effects of nutritional restriction on isotopic fractionation may be dampened or nullified by physiological and metabolic compensatory responses in species adapted to variable rates of food intake. Puffin nestlings have the physiological capacity to modulate metabolic rates in response to nutritional limitation (Kitaysky 1999). Furthermore, free-living puffin nestlings are fed diets rich in high-quality protein and growth may therefore be limited by energetic constraints, rather than by protein intake. In this paper, we report the results of two captive-feeding experiments in which we subjected puffin nestlings to nutritional deprivation during growth and measured changes in either whole blood or blood cell concentrations of $\delta^{13}C$ and $\delta^{15}N$.

1.3 Methods

We conducted two separate experiments to determine the effects of nutritional restriction on carbon and nitrogen isotopic fractionation in growing tufted puffin nestlings. In the first experiment, we utilized free-living nestlings reared in their burrows on Cliff Island in Chiniak Bay on Kodiak Island, AK, USA. We located 13 free-living puffin nestlings during the early stages of chick-rearing and excavated vertical access

holes which were patched with flat rocks to permit later access to the nesting chamber. When chicks were estimated to be approximately ten days old, based on wing chord measurements (Gjerdrum 2001), we blocked burrow entrances to prevent adults from provisioning their young and began feeding them one meal per day. Prior to the experiment, chicks were fed primarily capelin (*Mallotus villosus*) and Pacific sandlance (*Ammodytes hexapterus*) by their parents (Williams, unpublished data). We fed nestlings either 120g/day (control group; 650 kJ/day, n = 6) or 60g/day (experimental group; 325 kJ/day, n = 7) of Pacific herring (*Clupea pallasii*), plus a multivitamin supplement. Quantities of fish fed were determined based on a previous captive study (Kitaysky et al. 2005). Blood samples were collected and nestlings were weighed (±2 g) prior to feeding at ages 10, 19, 28, and 37 days. Blood was collected in heparinized 250-µl Natelson blood-collecting tubes and transferred into 1.5-ml microcentrifuge tubes which were stored on ice until frozen as whole blood samples at -20 °C until analysis. Following collection of the final blood sample, we fed all chicks ad libitum until they reached the age and size of wild fledglings, at which point we removed obstructions from the burrow entrance and allowed them to fledge on their own initiative or released them to the water.

In the second experiment, 14 puffins were reared from hatch in individual nest boxes under thermoneutral conditions with food provided twice daily in dishes placed on the bottom of nest boxes. For the duration of the experiment they were fed exclusively capelin. Nestlings were fed an ad libitum diet until they were ten days old, and then were randomly assigned to one of two diet regimes. The experimental group received 50g/day (270 kJ/day metabolizable energy) of capelin and the control group received 110g/day (594 kJ/day). When nestlings approached fledging age (means ± SE: control group = 49.6 ± 1.4 days; experimental group = 57.3 ± 0.6 days), they became restless and jumped from their nest boxes at night. When an individual jumped from their nest box during two consecutive nights, they were housed in a common area with access to a small pool of fresh water and ad libitum capelin supplied twice daily until the end of the experiment. Nestlings were weighed (±0.1 g) every five days beginning at hatch. Blood samples were

collected from post-absorptive nestlings by puncture of the alar vein with a 26g needle at ages 7, 14, 28, 42, and 75 days. Blood was collected in heparinized 100µl hematocrit tubes, transferred into 0.5-ml vials, and stored at 4 °C. Within 2h of collection, samples were centrifuged, plasma removed, and blood cells stored at -20 °C until analysis.

Ten herring from the batch fed to puffin chicks in the first experiment were collected for analysis of carbon and nitrogen stable isotopes. We also collected ten capelin and thirteen sandlance that were delivered by adult puffins to their chicks at another colony in Chiniak Bay for stable isotope analysis. Capelin used in the second experiment was from a single batch caught commercially in the Atlantic, but none was archived for analysis of stable isotopes. Prior to stable isotope analysis, lipids were extracted from prey samples using a Soxtech apparatus with chloroform solvent. Lipids were not extracted from whole blood or blood cells. All samples were freeze-dried and analyzed using a Costech Elemental Analyzer (ESC 4010; Valencia, CA, USA), and Finnigan MAT (San Jose, CA, USA) Conflo III interface with a Delta +XP Mass Spectrometer. Replicate measurements of internal laboratory standards indicated measurement errors to be ±0.20‰ for N, ±0.15‰ for C, and ±0.06 for the C:N ratio. Stable isotope concentrations are reported using "δ" notation according to $\delta X = [(R_{sample}/R_{standard})-1] \times 1{,}000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Standard values are based on atmospheric N_2 for $\delta^{15}N$, and the Vienna Peedee Belemnite (VPDB) for $\delta^{13}C$.

We performed all statistical analyses using SAS 9.1 (SAS Institute, Cary, NC, USA) and present data as means ± SE. In both experiments, separate repeated-measures mixed models (PROC MIXED) were used to determine the effects of nutritional regime, age, and their interaction on $\delta^{15}N$, $\delta^{13}C$, C:N ratio and mass. Use of repeated-measures mixed procedures allowed us to model the covariance structure of each data set to account for unevenly spaced sampling dates (Littell et al. 1998). Simple effects tests (LSMEANS/SLICE) were used to examine significant two-way interactions A x B (i.e., treatment x nestling age). This procedure tests for effects of A for each B, which is

calculated by extracting the appropriate row from the coefficient matrix for the A x B LSMEANS and using it to form an *F*-test. Finally, we estimate effect sizes of nutritional restriction on mass, $\delta^{15}N$, and $\delta^{13}C$ as the mean difference between treatment groups at ages 37 and 42 days in the first and second experiment, respectively.

1.4 Results

1.4.1 Experiment 1: Diet switched to herring at age ten days

Food restriction severely decreased rate of mass growth (Fig. 1.1a); mass was significantly affected by age ($F_{3,33}$=601.09, p<0.0001), treatment ($F_{1,11}$=212.77 , p<0.0001), and the interaction between age and treatment ($F_{3,33}$=109.90 , p<0.0001). At age 37 days, the mean difference in body mass between food restricted and control chicks was 227.9 g (95% CI: 205.3, 250.2). Food-restricted chicks were depleted in ^{15}N relative to control chicks (Fig 1.1b); values of $\delta^{15}N$ were significantly affected by age ($F_{3,33}$=20.30, p<0.0001) and treatment ($F_{1,11}$=22.05 , p<0.001; age x treatment $F_{3,33}$=1.04, p=0.39). Blood $\delta^{13}C$ values were also lower in food-restricted chicks compared to control chicks (Fig. 1.1c); $\delta^{13}C$ was affected by age ($F_{3,11}$=20.41, p<0.0001), but not by treatment ($F_{1,11}$=1.82, p=0.20). However, the interaction between age and treatment was significant ($F_{3,11}$=4.80, p=0.02). Post hoc effects tests indicated control chicks had significantly higher $\delta^{13}C$ values at ages 19, 28, and 37 days, but there was no significant difference between groups on the day of the diet switch (p>0.20). At age 37 days, the effect sizes of restriction on $\delta^{15}N$ and $\delta^{13}C$ values were 0.77‰ (95% CI: 0.29, 1.25) and 0.20‰ (95% CI: 0.09, 0.31), respectively. The ratio of carbon to nitrogen was not affected by age, treatment, or the interaction between age and treatment (p>0.50 for all). The mean C:N ratio of whole blood samples was 3.4 ± 0.2 (SE).

The mean $\delta^{15}N$ values of sandlance and capelin fed by adult puffins provisioning their young in Chiniak bay were 11.41 ± 0.18 (SE) and 11.41 ± 0.13, respectively. The mean $\delta^{13}C$ values of sandlance and capelin fed to puffin chicks by their parents were −18.49 ± 0.19 and −17.72 ± 0.21. The mean $\delta^{15}N$ value of herring fed to chicks was

12.52 ± 0.26 and the mean $\delta^{13}C$ value was −17.18 ± 0.15. At age 37 days, calculated diet-whole blood fractionation factors for nitrogen ($\Delta^{15}N$) were 2.28‰ (95% CI: 1.55, 3.02) and 3.05‰ (95% CI: 2.28, 3.82) for the restricted and control groups, respectively. Diet-whole blood fractionation factors for carbon ($\Delta^{13}C$) at age 37 days were −0.50‰ (95% CI: −0.89, −0.11) and −0.30‰ (95% CI, −0.72, 0.12) for restricted and control groups, respectively.

1.4.2 Experiment 2: Nestlings fed capelin from hatch

Food restriction severely decreased rate of mass growth (Fig. 1.2a); mass was significantly affected by age ($F_{13,156}$=129.62, p<0.0001), treatment ($F_{1,12}$=63.81, p<0.0001), and the interaction between age and treatment ($F_{13,156}$=6.69, p<0.0001). Effects tests revealed significant differences between treatment groups by age 20 days. At age 40 days (30 days of restriction) the effect size of restriction on body mass was 166.7g (95% CI: 146.4, 187.0). Blood cells of food-restricted chicks were depleted in ^{15}N relative to control chicks (Fig. 1.2b); $\delta^{15}N$ was significantly affected by age ($F_{4,48}$=83.39, p<0.0001) and treatment ($F_{1,12}$=8.47, p=0.013). The age and treatment interaction was not significant ($F_{4,48}$=2.47, p=0.057). Nutritionally restricted chicks had blood cells depleted in ^{13}C compared to control chicks (Fig. 1.2c); stable isotopes of carbon were significantly affected by age ($F_{4,12}$=15.63, p=0.0001), but not by treatment ($F_{1,12}$=4.47, p=0.056). However, $\delta^{13}C$ values were significantly affected by the interaction between age and treatment ($F_{4,12}$=24.59, p<0.0001). Post hoc effects tests revealed significant differences in $\delta^{13}C$ values between control and experimental animals at ages 28 and 42 days. At age 42 days (32 days of restriction), the effect sizes of restriction on $\delta^{15}N$ and $\delta^{13}C$ values were 0.41‰ (95% CI: 0.18, 0.64) and 0.25‰ (95% CI: 0.19, 0.32), respectively. At age 75 days (~18 days after restriction ended), there was no difference in $\delta^{15}N$ or $\delta^{13}C$ values between control and treatment groups. The C:N ratio of blood cells was affected by age ($F_{4,48}$=7.54, p<0.0001), but not by treatment ($F_{1,12}$=0.08, p=0.779; age x treatment: $F_{4,48}$=0.59, p=0.671). The C:N ratio of blood cells was slightly, but significantly, higher

at age seven days (mean ± SE: 3.34 ± 0.03) and then did not change for the remainder of the experiment (3.25 ± 0.01).

1.5 Discussion

1.5.1 Nitrogen stable isotopes

Whereas studies of fasting animals support the hypothesis that tissues become enriched in ^{15}N following nutritional restriction (Hobson et al. 1993, Oelbermann and Scheu 2002, Cherel et al. 2005a), we found whole blood and blood cells from nutritionally restricted puffin nestlings were significantly depleted in ^{15}N compared to well-fed conspecifics. Our study is not the first to question the effects of nutritional restriction on $\delta^{15}N$ values of consumer tissues. Studies of ectothermic invertebrates have failed to detect changes in whole-animal $\delta^{15}N$ values (Frazer et al. 1997, Schmidt et al. 1999) following several months of fasting. Ben-David et al. (1999) did not find any relationship between body condition and $\delta^{15}N$ in free-living arctic ground squirrels (*Spermophilus parryii plesius*), although they acknowledge that physiological effects may have been masked by ecological processes such as diet selection. Additionally, changes in body condition are sometimes attributed primarily to catabolism of fat stores (Niizuma et al. 2002) and enrichment of ^{15}N in body tissues is only expected if poor body condition is associated with protein loss (Martinez Del Rio and Wolf 2005).

Nutrient dynamics are complicated during growth as nutritionally restricted birds may be simultaneously protein-limited and in positive nitrogen balance. Kempster et al. (2007) found that a 35% reduction in food intake had no effect on $\delta^{15}N$ values of tissues in song sparrows (*Melospiza melodia*). Contrary to our study and to that of Kempster et al. (2007), Hobson et al. (1993) determined that food-restricted Japanese quail (*Coturnix japonica*) chicks become enriched in ^{15}N compared to growing birds fed ad libitum. The degree of nutritional restriction may explain the apparently contradictory results: chicks in the study of Hobson et al. (1993) were restricted to the extent that they did not gain body mass, whereas nestlings in Kempster et al. (2007) and in our study continued to

grow, albeit at a greatly reduced rate. Protein content of the diet may also play a role and was likely much higher in our study than in that of Hobson et al. (1993): quail chicks were fed commercial turkey starter, whereas puffins were fed a fish diet. With a decrease in content of dietary protein, the ratio of nitrogen assimilation to nitrogen loss increases and thus diet-tissue fractionation of nitrogen stable isotopes is predicted to decrease (Pearson et al. 2003, Martinez Del Rio and Wolf 2005). However, when exogenous amino acids are insufficient to meet demands for protein synthesis, recycling of metabolic amino acids should increase, producing the opposite effect (Voigt and Matt 2004). Taken further, food restriction may also produce an increase in diet-tissue fractionation of nitrogen isotopes in cases where growth is limited by dietary protein rather than energy intake.

Finally, species-specific differences in physiological response to nutritional restriction may be responsible for differences between studies. Puffin chicks are adapted to intermittent provisioning and are able to adjust their rates of development in response to food deprivation (Kitaysky 1999). Puffin nestlings subjected to nutritional restriction also down-modulate the hypothalamus-pituitary-adrenal (HPA) axis, resulting in decreased plasma levels of corticosterone (Kitaysky et al. 2005), an anti-anabolic stress steroid that functions to mobilize stored protein reserves. In contrast, the nutritional state of other seabird chicks is negatively correlated with activity of the HPA axis (Nunez-de la Mora et al. 1996, Kitaysky et al. 2001). Thus, food-deprived puffin chicks are able to spare endogenous protein stores and maintain a positive nitrogen balance despite a 50% reduction in food intake, which may explain the lack of ^{15}N enrichment in restricted chicks. However, song sparrow chicks subject to moderate food restriction exhibited numerous symptoms of nutritional stress, including elevated levels of corticosterone, yet $\delta^{15}N$ values were unaffected (Kempster et al. 2007).

Although we found significantly higher $\delta^{15}N$ values in well-fed (control) chicks compared to food-restricted animals, we do not believe that this was due to nutritional restriction *per se*. It is possible that this effect is due to incomplete turnover of blood cells

from hatch and slower turnover in restricted birds. However, this seems unlikely given $\delta^{15}N$ values of restricted chicks appeared to be approaching a different asymptotic level compared to control birds (Fig. 1.2b). Instead, we suggest the increase in $\delta^{15}N$ with body mass observed in our study is an artifact of growth and results from recycling of a continually expanding pool of metabolic nitrogen and/or from changes in nitrogen use efficiency. During growth, the size of endogenous protein stores and metabolic amino acid pools increased, while the amount of exogenous protein available remained unchanged. The metabolic amino acid pool is enriched relative to diet, so a proportional increase in amino acids drawn from the metabolic pool would produce progressive whole-body enrichment as body size increases. Additionally, growth of control chicks slowed as chicks aged, whereas food intake did not change, indicating protein was being used as a metabolic substrate to a greater extent during the latter half of the nestling stage. Thus, the ratio of nitrogen assimilation to nitrogen loss decreased as well-fed chicks grew, which is predicted to result in higher diet-tissue fractionation (Martinez Del Rio and Wolf 2005). Finally, the specific balance of essential amino acids needed for protein accretion differs from that needed to replace obligatory losses (reviewed in Klasing 1998), although the effect this has on diet-tissue fractionation is unknown.

1.5.2 Carbon stable isotopes

Although nutritional status is thought to primarily affect diet-tissue fractionation of nitrogen, a small and variable diet-tissue fractionation of ^{13}C occurs (see references in Kelly 2000), yet the cause of variability is not well understood. Hatch et al. (1995) determined that protein lysate (95% hemoglobin) from growing roosters and adult hens subjected to nutritional restriction became enriched in $\delta^{13}C$ and suggested its use as an indicator of catabolic state. Contrary to these findings, Hobson et al. (1993) found food restriction had no effect on $\delta^{13}C$ in muscle and liver tissue of quail chicks and fasting geese, whereas Cherel et al. (2005a) found ^{13}C was depleted in blood plasma, but not cells, from fasted birds.

Body lipids are depleted in ^{13}C relative to proteins and carbohydrates (DeNiro and Epstein 1977). Effects of food deprivation on the lipid content of tissues may consequently produce lower $\delta^{13}C$ values in lipid-rich tissues with a higher C:N ratio (Cherel et al. 2005a). However, in our second experiment, $\delta^{13}C$ values of blood cells were affected by nutritional restriction even though they have very little lipid content. Furthermore, the C:N ratios of whole blood and blood cells were unaffected by treatment, yet birds became progressively enriched in ^{13}C when fed ad libitum. Increased metabolism of exogenous or endogenous lipids associated with nutritional restriction will alter diet-tissue fractionation if carbon isotopes from these lipids are incorporated into other molecules during anabolic processes. The isotopic composition of body protein generally reflects the composition of dietary protein (Ambrose and Norr 1993), a process known as isotopic routing. However, carbon from oxidized fatty acids may be incorporated into the carbon skeletons of some nonessential amino acids because they are synthesized from intermediates of the carboxylic acid cycle (Klasing 1998). Thompson et al. (2000) suggested that incorporation of carbon from ^{13}C depleted lipids into proteinaceous tissues was responsible for the high variability in $\delta^{13}C$ signatures they observed between several free-living species of albatross. Cherel et al. (2005b) hypothesized this mechanism was responsible for variability in diet-tissue fractionation observed in captive penguins. Furthermore, by manipulating the concentrations and isotopic signatures of macronutrients in the diets of song birds, Podlesak and McWilliams (2006) determined that birds fed a low-protein diet incorporated dietary carbon from other macronutrients into proteins to a greater extent. We hypothesize that nutritional restriction may increase the amount of carbon from ^{13}C-depleted lipids incorporated into proteins as dietary and endogenous lipids are metabolized to meet energetic demands.

1.5.3 Tissue turnover

One limitation in our experiments is effects of growth and tissue turnover are necessarily confounded because both processes are occurring simultaneously.

Furthermore, observed isotopic turnover in growing animals reflects a combination of metabolic turnover and accretion of new tissue. Although turnover rates of tissues in slow-growing chicks are expected to be slower than control chicks, the asymptotic levels of $\delta^{15}N$ and $\delta^{13}C$ both appear to be different between treatment groups in both experiments, indicating turnover alone cannot explain the observed pattern. We selected whole blood and blood cells for isotopic analyses because these tissues are commonly used in ecological studies. However, use of plasma, a tissue with a much higher turnover rate than blood cells (Hobson and Clark 1993), may have allowed us to disentangle growth and turnover effects. Future studies may benefit by selecting plasma as a target tissue; however, lipids must be extracted prior to analysis because ^{13}C-depleted triglycerides are affected by nutritional restriction (Alonso-Alvarez and Ferrer 2001).

1.5.4 Implications for ecological studies

In this study, we tested the hypothesis that nutritional restriction affects $\delta^{15}N$ and $\delta^{13}C$ concentrations of blood in growing seabird chicks. Our primary objective was to determine if variation in nestling growth rates observed in wild populations would potentially confound estimates of trophic level of feeding and/or foraging location based on stable isotopes. Therefore, it is critical to first assess whether levels of food supplied to chicks in our experiments are comparable to food provisioned in wild populations. In our study, mass gain of nestlings during the "linear growth phase" (Gjerdrum 2001), was 17.15 g/day (control) versus 6.55 g/day (restricted) in the first experiment and 13.16 g/day versus 7.59 g/day in the second experiment. Piatt and Kitaysky (2002) review 49 colony years of nestling growth data and report an overall mean growth rate of 10.9 g/day ± 4.7 SD (range: −0.6 to 19 g/day). Thus, growth rates of our food restricted birds in our experiments were less than 1 SD below the overall mean and well within the range reported for wild birds. Control groups in our experiments grew faster than average, and in our first experiment, approached the maximum growth rate observed in the wild. At 38-40 days old, the minimum age required to fledge, masses of control and restricted

chicks in both experiments were within the range of fledging masses reported for wild birds (range 270-609 g; overall mean: 477 g ± 99.2 SD; Piatt and Kitaysky 2002). Thus, we conclude levels of food supplied to chicks in our experiments are within the range received by free-living nestlings.

The effect size of nutritional restriction on $\delta^{15}N$ in our experiments ranged from 0.41 to 0.77‰. Assuming $\delta^{15}N$ increases 3.4-3.8‰ per trophic level in marine systems (Minigawa and Wada 1984, Hobson and Welch 1992), nutritional restriction during growth would produce a maximum error in estimates of trophic level of approximately 0.23 trophic levels. The mean effect size of nutritional restriction on $\delta^{13}C$ was also relatively small in our study (0.20-0.25‰). Hobson et al. (1994) found $\delta^{13}C$ values from tissues of seabird species known to forage inshore versus offshore differed by 2-3‰. Therefore, despite effects of nutritional restriction on diet-tissue fractionation, stable isotopes of nitrogen and carbon should still provide a useful measure of trophic level of feeding and foraging location in free-living animals. Nevertheless, our results demonstrate that physiological processes affect stable isotope signatures and we caution isotope ecologists to consider these effects to avoid drawing spurious conclusions.

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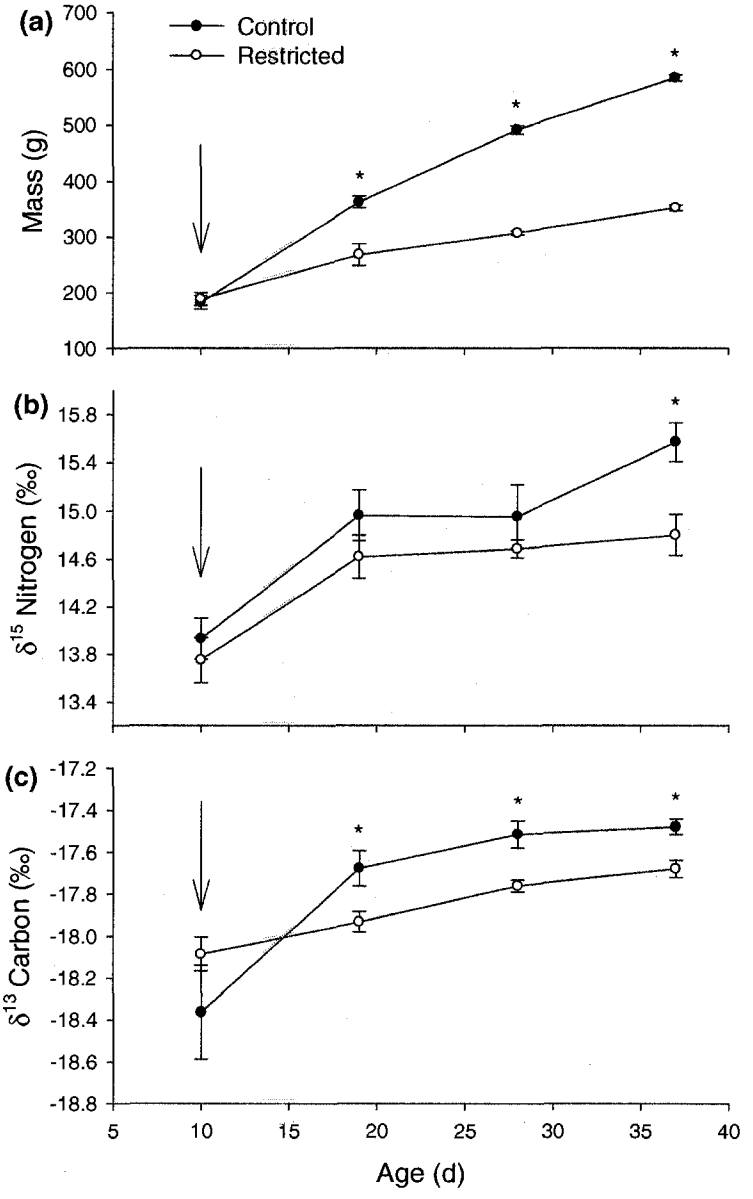

Fig. 1.1a-c Changes in the values of **a** mass, **b** whole blood $\delta^{15}N$, and **c** whole blood $\delta^{13}C$ (mean ± 1 SE) of tufted puffins (*Fratercula cirrhata*) with age in experiment 1 for control (650 kJ per day, n = 6, *filled circles*) and restricted diet (325 kJ per day, n = 7, *open circles*). Nestlings in this experiment were fed primarily capelin and sandlance by their parents until age ten days and then switched to a diet consisting exclusively of herring for the duration of the experiment. *Downward arrows* indicate implementation of nutritional restriction and diet switch. *Asterisks* indicate a significant difference (*F*-test, $P<0.05$) between control and treatment groups at a given age

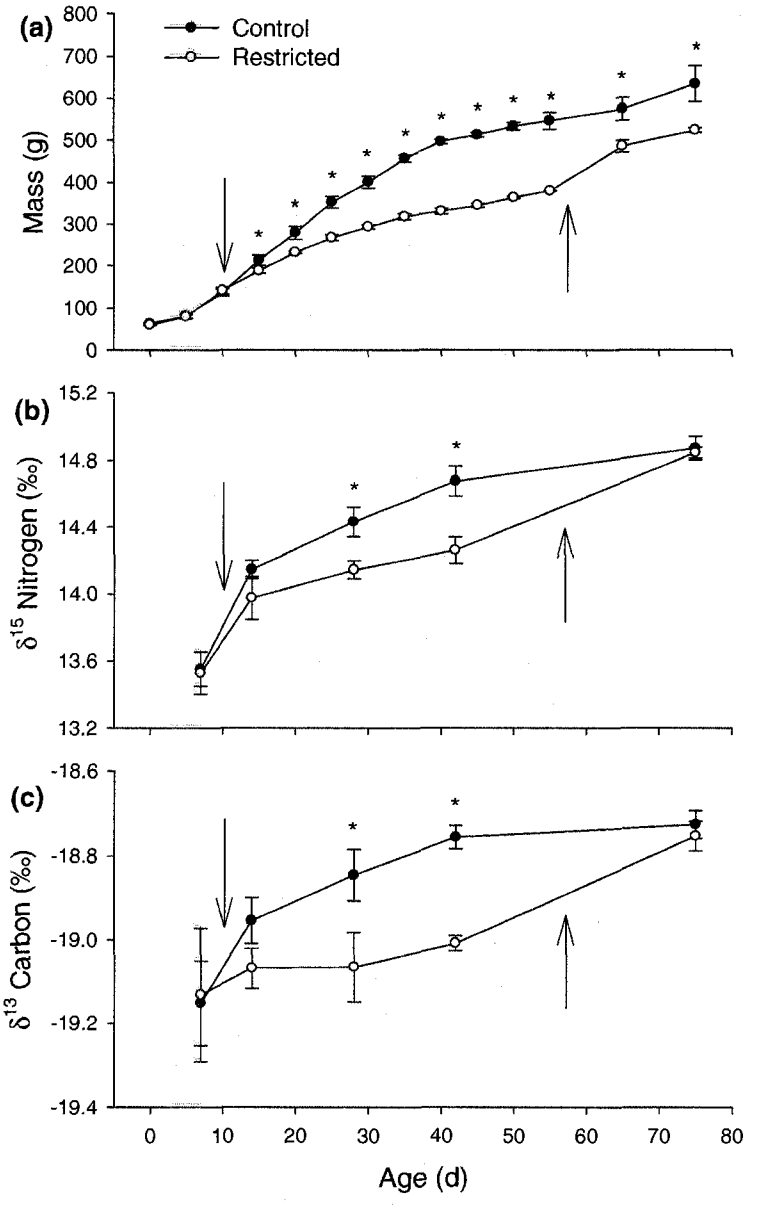

Fig. 1.2a-c Changes in the values of **a** mass, **b** blood cell $\delta^{15}N$ and **c** blood cell $\delta^{13}C$ (mean ± 1 SE) with age in experiment 2 for control (594 kJ per day, n = 7, *filled circles*) and restricted diet (270 kJ per day, n = 7, *open circles*). Chicks in this experiment were fed exclusively capelin from hatch. *Downward arrows* indicate implementation of nutritional restriction and *upward arrows* indicate the mean age that nestlings resumed an ad libitum diet regimen. Asterisks indicate a significant difference (*F*-test, $P<0.05$) between control and treatment groups at a given age

Chapter 2: Dietary restriction affects adipose tissue fatty acid signatures of tufted puffin (*Fratercula cirrhata*) nestlings[1]

2.1 Abstract

Fatty acid (FA) signature analysis is a powerful tool to investigate foraging ecology and food web dynamics in marine ecosystems. However, use of FA signatures to qualitatively or quantitatively infer diets is potentially confounded by effects of nutritional state on lipid metabolism. Estimation of diets using the quantitative fatty acid signature analysis (QFASA) model requires the use of calibration coefficients to account for predator metabolism of individual FAs. We conducted a captive feeding experiment to determine the effects of a 50% reduction in food intake on adipose tissue FA signatures of tufted puffin (*Fratercula cirrhata*) nestlings, a species that routinely experiences food restriction during growth. Following a shift in diet, FA signatures of chicks fed low- and high-calorie diets exhibited a similar change in composition. Rates of FA turnover were not different between high and low-calorie treatments and turnover was close to, but not entirely complete after 27 days on the controlled diet. Although FA signatures of tufted puffin nestlings were significantly affected by caloric restriction, calibration coefficients differed little between puffins fed low and high calorie diets. Our results demonstrate that changes in physiological state can affect FA metabolism but more research is required to better understand whether the size of these effects is sufficient to substantially alter diet estimation using the QFASA model.

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2.2 Introduction

Analysis of fatty acids (FAs) is increasingly being used to study foraging ecology and food web dynamics in marine ecosystems (reviewed in Budge et al., 2006). FAs are most commonly used in a qualitative manner to assess spatial and/or temporal differences in diet (Iverson et al., 1997; Walton et al., 2000) or to confirm the importance of certain prey types in the diets of marine predators (Raclot et al., 1998; Käkelä et al., 2006; Budge et al., 2007). FAs may also be used to quantitatively estimate predator diets using a statistical model to determine the combination of prey FA signatures that comes closest to matching the predator FA signature after accounting for effects of lipid metabolism (e.g. the QFASA method; Iverson et al., 2004). The QFASA method uses calibration coefficients to account for predator lipid metabolism by weighting individual FAs according to their tissue deposition relative to diet (Iverson et al., 2004, 2007). However, interpretation of FA data in studies of animal ecology is potentially complicated by factors that affect rates of biosynthesis, deposition, and metabolism of specific FAs within the predator.

Nutritional state, for example, has the potential to affect biochemical pathways and physiological processes, thus altering predator FA signatures. Numerous free-living animals routinely experience changes in nutritional state associated with different life-history stages including hyperphagia prior to migration, loss of body mass associated with reproduction, and periodic fasting associated with breeding, molt, or migration (Moreno, 1989; Barlein, 2002). However, studies examining whether these predictable changes in physiological state alter the FA composition of endogenous lipid stores are lacking. In addition to shifts in nutritional status associated with predictable life-history events, marine predators, including seabirds, may experience unpredictable changes in nutritional state associated with changes in prey availability. For example, growth rates of tufted puffin (*Fratercula cirrhata*, hereafter: puffin) nestlings exhibit high levels of spatial and temporal variability (Piatt and Kitaysky, 2002).

Analysis of FA signatures may furnish useful information regarding mechanistic links between environmental variability and reproductive parameters of puffins. For example, FA signatures could be used in a qualitative manner to determine if diets of adults and/or nestlings are different in years characterized by low reproductive output. Estimating diets of puffin nestling using the QFASA method might offer insight into the importance of certain forage fishes in nestling diets or changes in the energy density of diets (e.g. Beck et al., 2007). Determination of diet quality would potentially be useful to evaluate the "junk food hypothesis" which postulates recent declines in apex predator populations are due to a shift in the quality of prey available (Rosen and Trites, 2000; Wanless et al., 2005). However, a better understanding of the consequences of energy intake on lipid metabolism is needed to ensure differences in FA signatures measured in free-living populations reflect dietary shifts rather than changes in physiological state.

In this paper, we report the results of a captive feeding experiment designed to examine the relationship between energy intake and FAs in growing puffin nestlings. We subjected puffin nestlings to moderate food deprivation (50 % of high calorie treatment), equivalent to what nestlings routinely experience in the wild, and measured changes in adipose tissue FA signatures. Because food restriction coincided with a shift in diet, we used a repeated sampling design to determine if observed effects were due to differences in rates of FA turnover or were a consequence of altered FA metabolism. We then calculated calibration coefficients for tufted puffins fed high and low calorie diets and compared these coefficients to those obtained for common murres (*Uria aalge*) in a previous captive study (Iverson et al., 2007).

2.3 Materials and methods

2.3.1 Experimental procedures

We conducted an experimental feeding trial using free-living puffin nestlings reared in their burrows on Cliff Island in Chiniak Bay, Kodiak, AK (57°40'N, 152°20W) in 2004. Tufted puffins are monogamous with both parents caring for a single chick

raised in a burrow excavated in the soil. We located 13 puffin nestlings during the early stages of chick-rearing and excavated vertical access holes which were patched with flat rocks to permit later access to the nesting chamber. When chicks were estimated to be approximately 10 days old, based on wing chord measurements, we blocked burrow entrances to prevent adults from provisioning their young and began feeding them one meal per day. We fed nestlings either 120g/day (control group; 650 kJ/day, n=6) or 60g/day (experimental group; 325 kJ/day, n=7) of Pacific herring (*Clupea pallasi*) plus a multivitamin supplement. We measured body mass using a pesola spring scale (±2g) and biopsied subcutaneous adipose tissue according to the methods of Iverson et al. (2007) on days 0, 9, 18, and 27 of the experimental trial by extracting approximately 100-300mg of adipose tissue from a <1cm long incision located at a site just anterior to the uropygial gland and 1cm lateral to the spine. We alternated sampling locations between the right and left side of the spine so that each location was sampled only twice. Samples of adipose tissue were immediately placed in chloroform containing 0.01% BHT (butylated hydroxytoluene) as an antioxidant and stored on ice until frozen at -30°C. Blood samples were also taken for analysis of nitrogen and carbon stable isotopes and these results are reported elsewhere (Williams et al., 2007). After the final biopsy, we fed all chicks ad libitum until they reached the age and size of wild fledglings at which point we removed obstructions from the burrow entrance and allowed them to fledge on their own initiative or released them to the water.

2.3.2 Diets of free-living puffin nestlings

Presumably, FA signatures measured in 10 day old chicks were reflective of diets fed by parents since hatch, although it is possible that there was a small residual influence of pre-hatch FA signatures at this stage. We did not determine diets of chicks prior to the experiment on Cliff Island because adult puffins are particularly susceptible to disturbance during incubation and early chick-rearing. However, we collected samples of chick meals at this colony by burrow screening (Hatch and Sanger, 1992) for five weeks

following initiation of the feeding trial. Because we only obtained 26 meal samples from Cliff Island during this period, we also report diets from burrows screened on Chiniak Island (n=158 meals), located 22 km east of Cliff Island. Diets likely differed somewhat between the two islands; however, we assume the dominant prey species fed to nestlings on Chiniak Island were the same as what chicks consumed on Cliff Island and that FA signatures of prey species would differ little between the two sites. We calculated the percentage composition by weight within each sample and report the mean percent diet composition for each island.

2.3.3 Laboratory analyses

We extracted lipids from adipose tissue samples and from prey samples according to Folch et al. (1957) as modified by Iverson et al. (2001). We used a subset of the prey items collected at both islands and supplemented this with nine juvenile Pacific cod (*Gadus macrocephalus*) and thirteen juvenile salmonids (*Onchorynchus spp.*) that were collected in beach seines within Chiniak Bay. FA methyl esters (FAMEs) were prepared from ≤100 mg of the lipid extracts using 3.0 ml Hilditch Reagent (0.5 N H_2SO_4 in methanol) in 1.5 ml methylene chloride with BHT, capped under nitrogen and heated at 100°C for one hour (Budge et al., 2006). We successfully obtained FAMEs for all samples with the exception of one biopsy from a 10 day old chick from the control group; the biopsy for this chick was too small to yield sufficient sample. Thin-layer chromatography revealed that FAMEs generated from some prey samples contained fatty alcohols due to the presence of wax esters. We transformed these alcohols into FAs using modified Jones' reagent (CrO_3 in H_2SO_4), as described by Budge and Iverson (2003), and repeated the transesterification using Hilditch reagent. Following transesterification, FAMEs were extracted into hexane, concentrated using nitrogen gas, and then brought up to a final volume of 50 mg FAME/ml hexane. Identification and quantification of FAMEs were performed in duplicate using temperature programmed gas-liquid chromatography on a Perkin Elmer Autosystem II Capillary FID gas chromatograph fitted with a 30m x

0.25 mm column coated with (50 % cyanopropyl) - methylpolysiloxane (DB-23) and linked to a computerized integration system (Tubochrom 4 software, PE Nelson, San Jose, CA). Each chromatogram was manually assessed for correct identification of peaks and reintegrated where necessary.

2.3.4 Calibration coefficients

An integral part of quantitative diet estimation using QFASA modeling is the development of calibration coefficients to account for the effects of lipid metabolism within the predator. We calculated calibration coefficients for tufted puffins in the low and high calorie groups after they had been on a constant herring diet for 27 days. The calibration coefficient of a particular FA is the ratio of the percentage composition of that FA in the adipose tissue of the puffin to the average percentage composition of that FA in herring (see details in Iverson et al. 2004).

2.3.5 Statistical analyses

We performed all statistical analyses using SAS 9.1 (SAS Institute, Cary, NC) and present data as means ± SE. Because the number of identified FAs (69) greatly exceeds the number of samples, we restricted our analyses to FAs in adipose tissue with overall means >1.0% of total FAs, excluding 22:5n-3, because it may be an intermediate of 20:5n-3 and 22:6n-3 (Ackman et al., 1988). The 12 FAs that met this criterion accounted for 88 % by mass of the total FAs in puffin adipose tissue. Percentages of the 12 FAs were renormalized over 100 % and then transformed into log ratios according to the following: $x_{trans} = \log (x_i/c_r)$ where x_i is the percentage of a given FA, x_{trans} is the transformed FA and c_r is 18:0, a reference FA (Budge et al., 2006). Because the log of zero cannot be taken, values of zero were changed to 0.005, a value considered to be below the minimum detection level of 0.01 %. Transformation of raw percentages into log ratios was done to break the constraint that each observation must sum to a constant (Aitchison, 1986). We performed principal component analysis (PCA) on the 11

transformed FAs and extracted the first and second principal components (PC1 and PC2). We used repeated-measures mixed models to determine the effects of age, treatment, and the interaction between age and treatment on PC1 and PC2. Use of repeated-measures mixed procedures allowed us to model the covariance structure of each data set and permitted inclusion the individual with the missing FA data (Littell et al., 1998).

We performed univariate and multivariate analyses on the prey data using the same 12 FAs previously selected for puffins. These FAs accounted for 85 % by mass of the total FAs. We performed a principal component analysis on the 11 transformed FA and extracted the first and second principal components. We then tested for differences between PC1 and PC2 using Kruskal-Wallis tests (ANOVA on ranks) followed by post-hoc Tukey HSD tests with alpha adjusted using a Bonferronni correction (e.g. alpha = 0.05/2 = 0.025). We also conducted univariate analyses on the 12 selected FAs; we used Kruskal-Wallis tests followed by Tukey HSD tests with alpha adjusted using a Bonferronni correction (alpha = 0.05/12 = 0.0042). Non-parametric Kruskal-Wallis tests were used because raw and log-ratio transformed data failed to meet the homogeneous variance assumption required for parametric methods. The Bonferronni correction controls the family-wise error rate but is extremely conservative and increases the likelihood of making a type II error.

2.4 Results

2.4.1 Diets of free-living puffin nestlings

We collected 28 meal samples comprised of 45 fish on Cliff Island and 158 meal samples comprised of 718 fish on Chiniak Island. Most meal samples collected from Cliff Island were likely only partial meals; eighteen of twenty-eight meal samples were comprised of a single fish. At both islands, diets were dominated by Pacific sandlance (*Ammodytes hexapterus*) and to a lesser extent, capelin (*Mallotus villosus*). Estimated diet composition based on burrow screening for nestlings at both islands is shown in Table 2.1.

2.4.2 Puffin FA signatures

Food restriction severely decreased rate of mass growth (Fig. 2.1a); mass was significantly affected by age ($F_{3,33}$=601.09, p<0.0001), treatment ($F_{1,11}$=212.77, p<0.0001), and the interaction between age and treatment ($F_{3,33}$=109.90, p<0.0001). We extracted two significant principal components from the PCA on transformed FAs; PC1 and PC2 accounted for 61% and 21% of the total variation in the twelve selected FAs, respectively. After the switch from fishes fed by parents to a herring diet, PC1 increased in both the restricted and control groups, but never achieved an asymptotic level in either group by the end of the experiment (Fig. 2.1b). PC1 was significantly affected by age ($F_{3,11}$=263.59, p<0.0001) and treatment ($F_{1,11}$=9.63, p=0.010). The interaction between age and treatment approached significance ($F_{3,11}$=3.20, p=0.066). PC2 only increased in the control group. PC2 was significantly affected by age ($F_{3,11}$=4.70, p=0.024) and treatment ($F_{1,11}$=15.86, p=0.002, Fig. 2.1c), but the interaction between age and treatment was not significant ($F_{3,11}$=1.45, p=0.28). Non-transformed values of the twelve most abundant FAs are shown in Fig. 2.2.

2.4.3 Prey FA signatures

The first and second principal components from a PCA of prey species using the 11 transformed fatty acids accounted for 60% and 16% of the total variation, respectively. PC1 differed between species ($F_{5,104}$=27.61, p<0.0001); post-hoc Tukey tests indicated no significant difference in PC1 between herring, sandlance, and capelin, but all three species differed (p<0.025) from sandfish, cod, and salmon, which formed a second group of fishes that were not significantly different from one another (Fig. 2.3). PC2 also differed between species ($F_{5,104}$=4.54, p<0.001); post-hoc Tukey tests indicated the mean PC2 value of cod was significantly different (p<0.025) from that of herring and salmonids. For univariate analyses, we found a significant difference between species (P<0.0042) for all 12 FAs with the exception of 18:1n-9. Post-hoc Tukey tests revealed

that each species differed from all other species in at least one of the twelve FAs (results shown in Fig. 2.4). FAs that were more abundant (measured as % total FAs) in herring compared to sandlance and capelin increased in puffin chicks following the diet switch whereas FAs that were less abundant in herring decreased in puffins.

2.4.4 Calibration coefficients

Calibration coefficients calculated for puffins fed a constant diet of herring differed little between high and low calorie treatment groups (Fig. 2.5). Calibration coefficients for puffins were also similar to values previously obtained for common murres, with the exception of 20 and 22 carbon length monounsaturated FAs. The silverside (*Menidia menidia*) fed to common murres had much lower levels of these FAs (Iverson et al., 2007).

2.5 Discussion

FA signatures of puffin nestlings fed high and low-calorie diets changed similarly following a switch from a diet that was presumably comprised primarily of sandlance and capelin to a diet comprised exclusively of herring. Turnover of FA signatures was close to, but not entirely, complete after 27 days on the controlled diet. However, based on the second principal component (PC2), we suggest that nutritional restriction affects adipose tissue FA signatures independent of turnover effects. Examination of individual FAs supports our inferences based on PCA: turnover was not entirely complete at the end of the experiment, yet FAs from food-restricted and control groups appeared to be approaching different asymptotic levels. Nevertheless, calibration coefficients calculated for QFASA modeling differed little between puffins fed high and low calorie diets. Most FA calibration coefficients calculated for puffins were also similar to coefficients calculated for common murres fed a silverside diet since hatch, although notable differences were found for 20 and 22 carbon length mono-unsaturated FAs.

An important consideration when manipulating energy intake in growing animals is determining whether experimental procedures effectively mimic natural conditions. In our study, mass gain of nestlings during the linear growth phase (age 10-30 d; Gjerdrum, 2001) in high and low-calorie groups was 17.15 g/day and 6.55 g/day, respectively. Piatt and Kitaysky (2002) review 49 colony years of nestling growth data and report an overall mean growth rate of 10.9 g/day ± 4.7 SD (range: –0.6 to 19 g/day). Thus, growth rates of food-restricted birds in our experiments were less than 1 SD below the overall mean and well within the range reported for wild birds. Growth rates of the high-calorie group approached the maximum level observed in the wild.

We used a repeated sampling design to determine whether observed effects were due to differences in turnover rates or to changes in the characteristics of lipid metabolism. We did not find a significant interaction between age and treatment for either PC1 or PC2, suggesting turnover was similar between groups, although our sample size was small. Turnover of FAs is a function of both metabolic turnover and accretion of additional fat stores during growth. We predicted greater accumulation of fat stores in chicks fed the high-calorie diet would have a dilution effect resulting in more rapid turnover. Oyan and Anker-Nilssen (1996) determined the amount of subcutaneous fat stored by Atlantic puffin (*Fratercula arctica*) chicks depends on caloric intake, yet we found no evidence for more rapid turnover in the high-calorie group. The apparent lack of an effect of energy intake on FA turnover may indicate food-restricted chicks were mobilizing fat stores to meet their energetic demands, resulting in a turnover rate similar to that of well-fed chicks.

In addition to dietary FA composition, predator FA signatures depend on 1) selective metabolism of ingested FAs, 2) modification of exogenous FAs through elongation and desaturation, 3) selective mobilization and/or deposition of stored FAs, and 4) de novo synthesis of FAs. Changes in nutritional state may alter FA signatures through any of these mechanisms. For example, rates of de novo synthesis decrease during fasting in neonatal chicks fed high-carbohydrate diets (e.g. Back et al., 1986) and

it is possible that lipogenesis in puffin chicks increased in response to a high calorie diet. In our study, four of the six FAs (14:0, 16:0, 16:1n-7, and 18:1n-7) that are likely to be synthesized de novo within puffins were less abundant (measured as % total FA) in food restricted puffins, which is consistent with the hypothesis that rates of de novo synthesis are positively correlated with nutritional state in growing chicks. The two remaining FAs (of the 12 selected) that are likely synthesized de novo (18:0 and 18:1n-9) did not show a consistent difference between high and low-calorie groups. Caloric restriction normally triggers an increase in FA synthesis in non-growing animals (e.g. Lee et al. 1999). Thus, more research is required to determine what the effects of nutritional state on de novo synthesis of FAs in rapidly-growing animals fed diets rich in protein and lipids.

Effects of nutritional state on adipose tissue FAs may occur through mechanisms other than de novo synthesis. Fasting in laboratory rats, for instance, alters adipose tissue FA signatures (Raclot and Groscolas, 1995) apparently through selective re-uptake of FAs during lipolysis (Raclot and Oudart, 2000). FAs affected in our feeding trial were generally not consistent with those affected in the rat experiments as we did not find evidence for greater mobilization of long-chain or poly-unsaturated FAs (PUFAs). However, we did find higher levels of 22:1n-1 and 20:1n-1 in restricted chicks and these two FAs were the least mobilized of the 17 FAs examined by Raclot and Groscolas (1995). In contrast, Iverson and Kirsch (unpublished data cited in Iverson et al., 2004) found no effect of fasting on blubber FA signatures in seals. Thus, it appears effects of nutritional state on FA signatures are likely to be taxon-specific.

Effects of nutritional restriction on the biochemical pathways responsible for modification of FAs via elongation and desaturation of FAs could also be important. In birds, dietary triacylglicerides are packaged into portomicrons prior to secretion into the portal vein and subsequent uptake by the liver. Within the liver, dietary lipids are repackaged with FAs synthesized de novo as very low density lipoproteins (VLDL) which are secreted into the blood (reviewed in Klasing, 1998). Because dietary lipids are routed through the liver in birds, it is possible that FAs are modified, via elongation and

desaturation, to a greater extent than in mammals. However, predicting the consequences of nutritional restriction on FA metabolism in piscivorous seabirds is difficult because within birds these pathways have only been studied extensively in Galliformes (poultry). Given the large amount of long-chain PUFAs available in their marine diet, it seems likely the capacity of seabirds to synthesize and modify certain PUFAs may be absent or greatly reduced. For example, the essential fatty acid requirements of freshwater and marine fish differ; marine fish have a dietary requirement for 20:4n-6, 20:5n-3, and 22:6n-3 because of deficiencies in one or more enzymes in the desaturation/elongation pathways from 18:2n-6 and 18:3n-3 (reviewed in Tocher, 2003).

Our results indicate FA signatures of adipose tissue can be affected by changes in nutritional state in growing seabirds. However, the size of these effects was small relative to the change in dietary FAs in our study. Thus, chicks fed low and high calorie diets exhibited a similar change in FA signatures following the diet switch. Additionally, calibration coefficients differed little between treatment groups. More work is needed to determine how robust diet estimation using the QFASA model is to the effects of nutritional state on lipid metabolism observed in our study. For example, Iverson et al. (2004) found that diet estimation based on QFASA modeling produced similar results when slightly different sets of calibration coefficients were used. Ultimately, error in diet estimation will depend on the degree of inter-specific variability in potential prey species being modeled. The similiarity between puffin and murre calibration coefficients suggests metabolim of ingested lipids is comparable between these two species of alcids. However, murre calibration coefficients were exceptionally high for 20-22 carbon length mono-unsaturated FAs (i.e. 20:1 and 22:1). This difference is likely due to the very low levels of these particular FAs in the silverside diet fed to murres (see Iverson et al., 2007).

Our experimental approach did not allow us to determine the mechanism(s) responsible for the observed effects and we encourage future studies using radiolabeled precursors (e.g. Budge et al., 2004) or stable isotopes (e.g. Su et al., 2001) to address this

issue. Ultimately, no method of diet determination will provide exact results and a combination of methods should be used whenever possible. Information on diets furnished from molecular techniques, such as analysis of stable isotope (e.g. Kelly, 2000), faecal DNA (e.g. Reed et al., 1997), and FAs, should be used to compliment, rather than replace, conventional techniques (e.g. stomach content analysis; Springer et al., 1996). Although FA signature analysis shows great potential for diet assessment in marine ecosystems, more controlled experiments are needed to identify potential caveats associated with the method.

2.6 Acknowledgements

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Table 2.1 Mean diet composition (% by mass) of tufted puffin nestlings on Cliff and Chiniak Islands, as estimated using the burrow screening method in August 2004.

Prey Species	Diet Composition (% by mass)	
	Cliff Island (n = 26 meals)	Chiniak Island (n = 158 meals)
Pacific sandlance *Ammodytes hexapteru*	76 %	65 %
Capelin *Mallotus villosus*	12 %	21 %
Pacific sandfish *Trichodon trichodon*	8 %	5 %
Pacific cod *Gadus macrocephalus*	0 %	4 %
Salmonid *Onchorynchus* spp.	4 %	3 %
Other	1 %	2 %

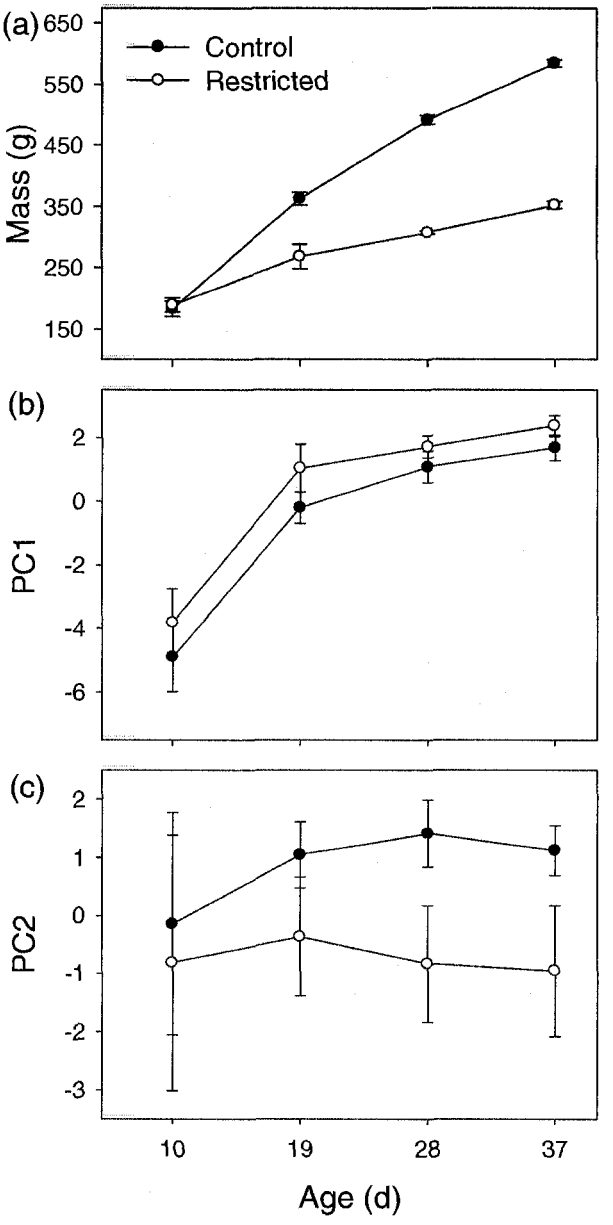

Fig. 2.1 Changes in the values of (a) mass, (b) PC1, and (c) PC2 (mean ± SE) of tufted puffins with age for high-calorie (650 kJ per day, n = 6, filled circles) and low-calorie diet (325 kJ per day, n = 7, open circles). PC1 and PC2 are the first and second principal components output from a PCA on transformed fatty acids (FAs). PC1 and PC2 account for 61 and 21% of the total variation in select FAs, respectively. Nestlings were fed by their parents until ten days of age and then switched to a diet consisting exclusively of herring for the duration of the experiment.

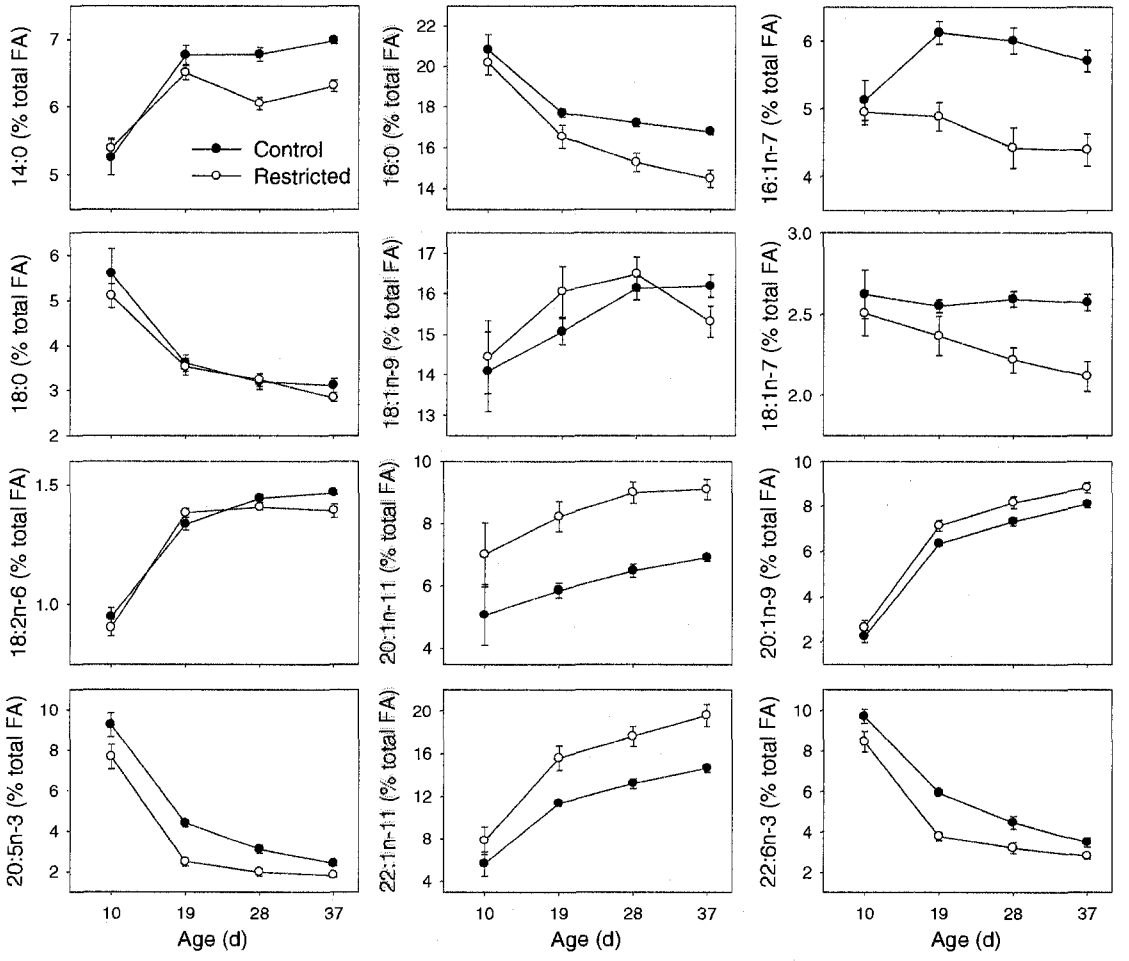

Fig. 2.2 Changes in the 12 most abundant fatty acids (mean ± SE) in adipose tissue of tufted puffins with age for high-calorie (650 kJ per day, n = 6, filled circles) and low-calorie diet (325 kJ per day, n = 7, open circles). Nestlings were fed by their parents until ten days of age and then switched to a diet consisting exclusively of herring for the duration of the experiment.

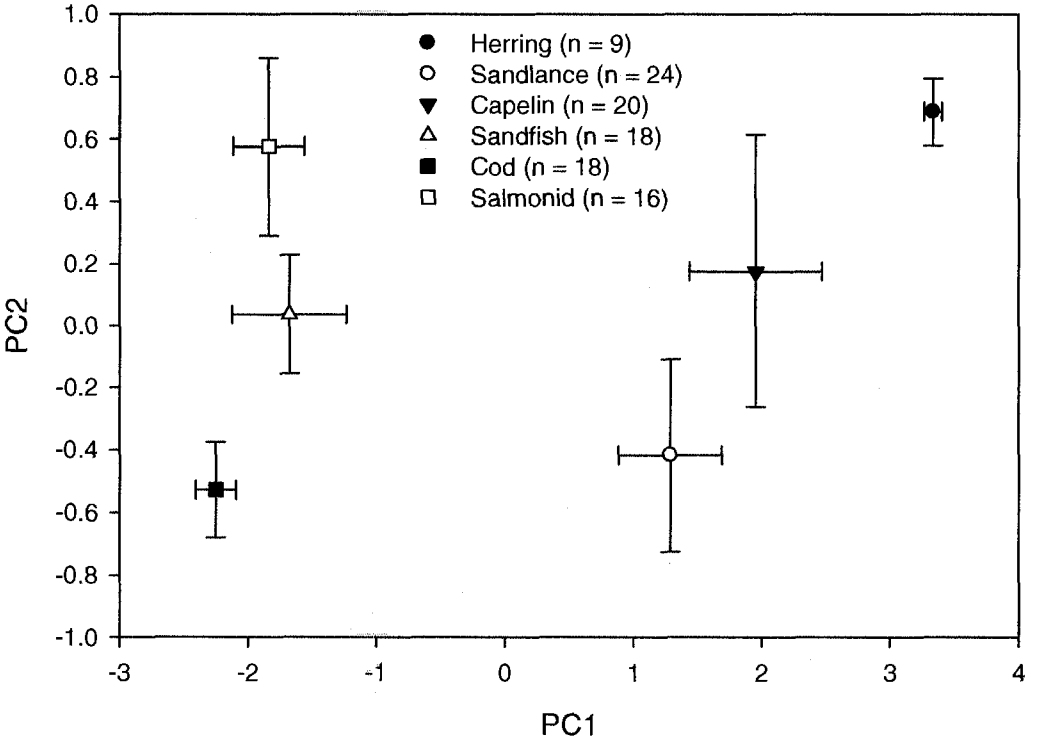

Fig. 2.3 Principal component analysis (PCA) of prey items fed to chicks in 2004. PCA was performed using transformed fatty acids. Herring was fed by hand to chicks in the experiment whereas other fishes were fed to nestlings by their parents at Cliff and Chiniak Islands. The first (PC1) and second (PC2) principal components accounted for 60 % and 16 % of the total variation, respectively. Values are mean ±SE.

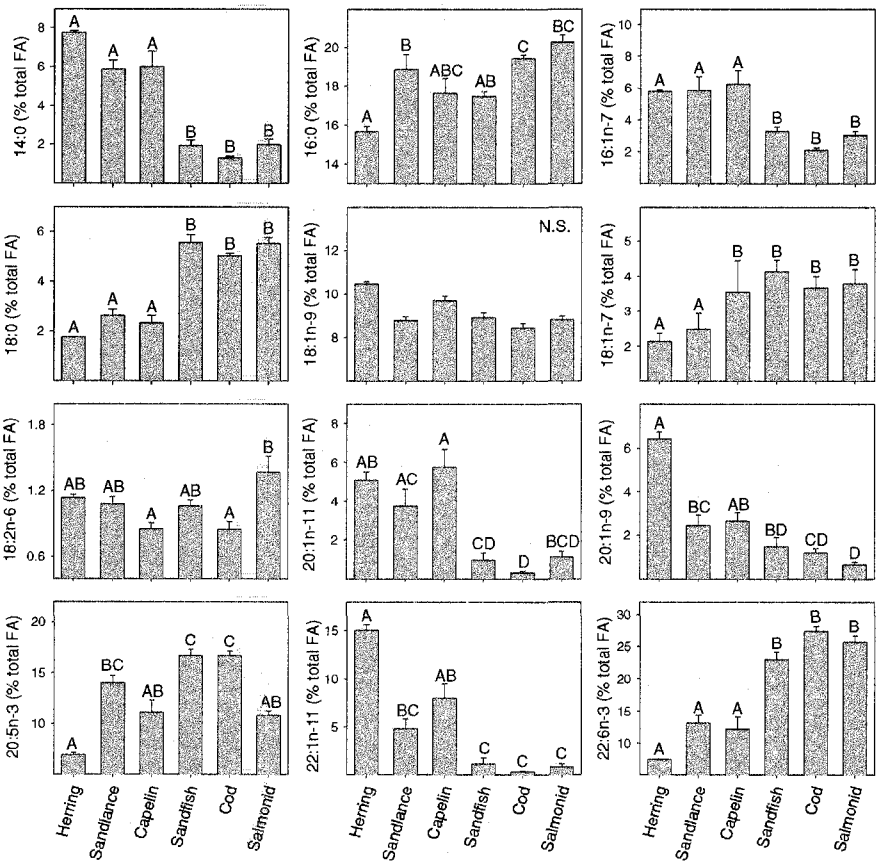

Fig. 2.4 The 12 most abundant fatty acids (FAs, mean ± SE) in prey items fed to tufted puffin nestlings in 2004. Herring was fed by hand to chicks in the experiment whereas other fishes were fed to nestlings by their parents on Cliff and Chiniak Islands. These 12 FAs represent the most abundant FAs measured in the adipose tissue of puffin nestlings in the experiment. In each panel, different letters indicate significant differences between species for that particular FA (Kruskal-Wallis tests followed by Tukey HSD tests with alpha adjusted using a Bonferronni correction. e.g. alpha = 0.05/12 = 0.0042). Sample sizes for each group are shown in Fig. 2.3

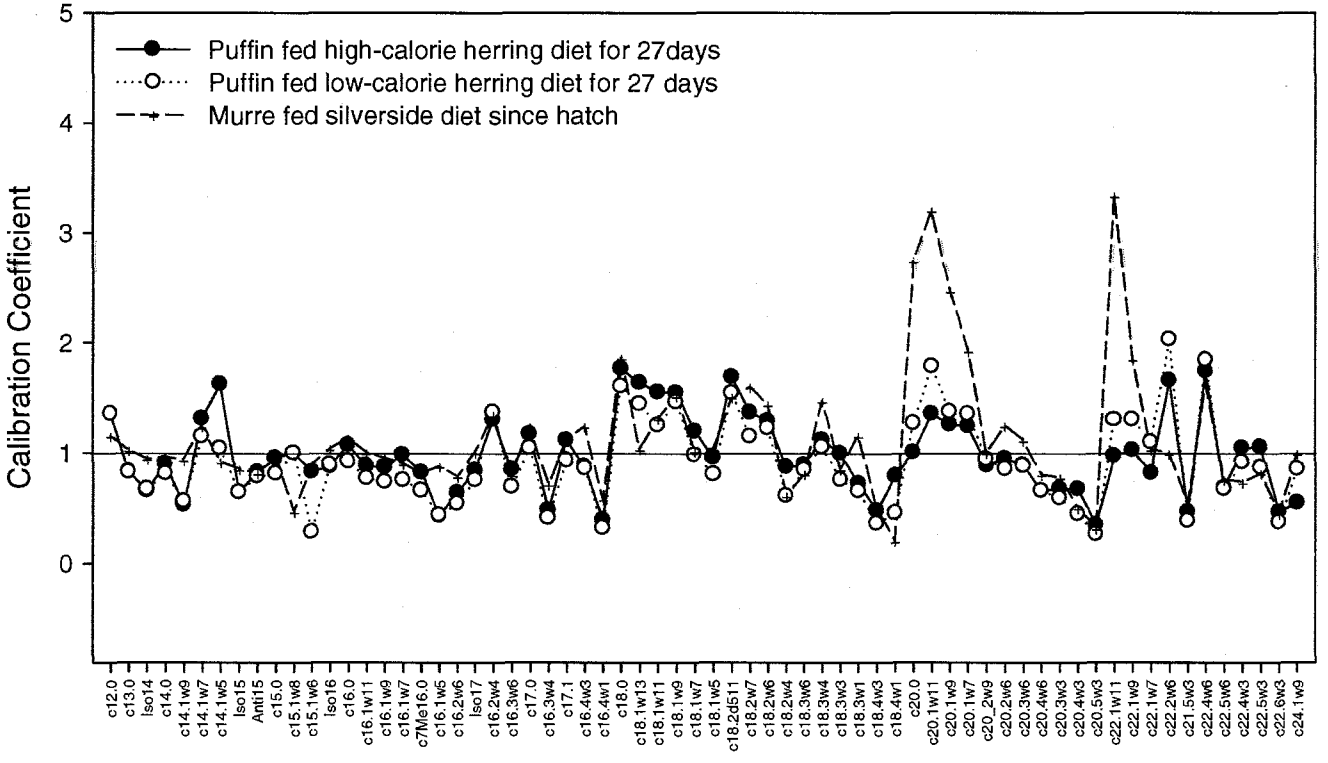

Fig. 2.5 Calibration coefficients for tufted puffins fed a high-calorie herring diet (650 kJ per day, filled circles) and a low-calorie herring diet (325 kJ per day, open circles). Calibration coefficients for common murres fed a diet of silverside (247 kJ per day, plus symbol) are shown for comparison (from Iverson et al. 2007).

Chapter 3: Stable isotopes and fatty acid signatures reveal age- and stage-dependent foraging niches in tufted puffins[1]

3.1 Abstract

Major breeding failures of seabird populations are sometimes attributed to reduced egg laying or abandonment of incubation due to nutritional stress, yet diets during these reproductive stages are often poorly characterized. We used stable isotopes and fatty acid (FA) signatures to infer age- (adult vs. nestling) and stage-dependent foraging niches of tufted puffins (*Fratercula cirrhata*) captured in Chiniak Bay, Kodiak Island, AK from 2003-05. Whole blood $\delta^{15}N$ values indicate a seasonal shift in trophic niche of adults: ^{15}N enrichment was consistent with a 0.47 to 0.68 increase in trophic level of feeding from pre-lay to late chick-rearing. Although incomplete turnover of blood cells from the pre-lay period likely contributed to intermediate $\delta^{15}N$ values of incubating puffins, this was insufficient to account for differences between incubating and chick-rearing adults. Differences in $\delta^{15}N$ and $\delta^{13}C$ between chick-rearing adults and nestlings were small and inconsistent between years. Discriminant function analysis (DFA) using the 14 most abundant FAs classified individuals to reproductive stage and age within each year with a high level of accuracy (linear DFA: 93-99%; quadratic DFA: 80-92%). When all years were combined, accuracy of cross-validated classification remained high (linear DFA: 90%; quadratic DFA: 76%). Based on stable isotopes and FA signatures, we conclude foraging niches are stage-dependent in this species and suggest chick-rearing adults do not typically feed at a lower trophic level than nestlings but likely consume a different array of prey species.

[1]Subsequent version published as: Williams CT, Iverson SJ, Buck CL (In Press) Stable isotopes and fatty acid signatures reveal age- and stage-dependent foraging niches in tufted puffins. Marine Ecology Progress Series.

3.2 Introduction

Marine food webs, fisheries, and apex predators are highly vulnerable to climate-driven changes in physical forcing that occur at a variety of spatial and temporal scales (Mann & Lazier 2006). Primary production and ecosystem dynamics in the North Pacific, for example, are affected by multi-year invasions of nutrient-poor warm surface waters associated with the El Nino Southern Oscillation (Zamon & Welch 2005) and by fluctuations between warm and cold regimes that occur on a 20-30 year cycle known as the Pacific Decadal Oscillation (Francis et al. 1998, McGowan et al. 1998). Oscillations in ocean climate conditions can have profound effects on seabirds and other upper-trophic level predators through alterations in the abundance and spatial, temporal, and age distributions of potential prey species (Anderson & Piatt 1999, Benson & Trites 2002).

Reproductive parameters of seabirds are often proposed as useful indicators of marine prey availability because they are easily observed and sensitive to changes in oceanographic conditions (Cairns 1987, Montevecchi 1993). In puffin species, for example, reproductive phenology and growth and survival rates of nestlings are correlated with sea surface temperatures (SSTs; Durant et al. 2003, Gjerdrum et al. 2003, Harding et al. 2003). Changes in SSTs presumably affect reproduction by altering the availability of key forage species (e.g. Durant et al. 2006). Moreover, because prey fed by adult puffins to their nestlings can be collected with relative ease, they have been touted as useful samplers of prey availability, providing information on ecologically important schooling forage fishes, as well as the early life-stages of several species of commercial importance (Hatch & Sanger 1992, Barrett 2002). However, identifying the mechanistic link between changes in ocean climate and reproductive parameters is problematic because diets of adult puffins during the breeding season are poorly characterized and information furnished from nestling diets is limited in temporal scope.

Although reproductive success in seabirds may be limited by the capacity of adults to obtain food for their young, breeding failure often occurs during incubation or prior to egg-laying (e.g. Chastel et al. 1995, Wanless et al. 2005). Diets of adult puffins are potentially affected by extrinsic factors, such as prey availability, as well as intrinsic

factors associated with constraints imposed by reproduction. Time-budgets of several species of seabirds, for example, vary depending on reproductive stage (Schaffer et al. 2003, Humphreys et al. 2006), which likely influences foraging distribution and consequently prey selection. During chick-rearing, adult puffins must return to the colony several times each day, which potentially limits their foraging range and access to certain habitats. Furthermore, it is sometimes assumed that adult seabirds consume the same prey as they provision to their young (Hatch & Sanger 1992); however, evidence from analyses of stomach contents (Baird 1990, Mehlum 2001, Piatt & Kitaysky 2002) and stable isotopes (Hobson et al. 2002) suggests this assumption may be invalid for some species. According to central place foraging theory, birds that transport prey in their bill should attempt to maximize the rate of energy provided to their offspring by selecting large, high quality prey items to feed their young (Orians & Pearson 1979). When feeding for self-maintenance, adults may increase their rate of energy intake by selecting smaller prey items that occur in highly predictable and dense aggregations (Baird 1991, Mehlum 2001). Thus, linking prey availability to reproductive success in seabirds requires methods that permit the determination of diet composition in adults throughout the breeding cycle.

Stomach content analysis of collected birds is conventionally used to assess puffin diets outside of the chick-rearing period (Baird 1990, Baird 1991, Piatt & Kitaysky 2002). Unfortunately, this technique only provides a snap-shot of what the bird has been eating immediately prior to collection and is subject to bias associated with the under-representation of soft bodied organisms and the retention of hard parts (Votier et al. 2003). Furthermore, it is difficult to avoid spatial sampling bias when assessing diets based on stomach contents because the distribution of foraging seabirds is usually unknown. Stomach contents of birds collected at the colony are likely biased towards prey consumed in proximate foraging habitat. However, molecular techniques such as fatty acid (FA) signature analysis and stable isotope analysis represent non-lethal alternatives that provide an integration of diet over a longer period and avoid such bias.

Use of stable isotope analysis as a means to provide an estimate of feeding at a specific trophic level is based on evidence that stable isotopes of nitrogen show a predictable stepwise enrichment with trophic level in marine systems (Minigawa & Wada 1984; Hobson & Welch 1992). This enrichment is due to discrimination against the heavier isotope (^{15}N) during transamination of amino acids, resulting in preferential excretion of the lighter isotope (Macko et al. 1986). Hobson et al. (1994) demonstrated that ^{15}N enrichment is useful to estimate relative, and possibly absolute, trophic level in seabirds whereas $\delta^{13}C$ is more suited to examining foraging distribution (inshore vs. offshore) and food web links (pelagic vs. benthic). FA signature analysis is based on the premise that FAs of carbon chain length $\geq$14 are deposited directly into adipose tissue with minimal modification, or in a predictable manner, permitting inferences on predator diets (Iverson 1993). Some selective metabolism of ingested FAs and biosynthesis of certain FAs produces FA signatures in the predator that will never directly match dietary signatures (Iverson et al. 2004, Budge et al. 2006). Nevertheless, effects of metabolism and biosynthesis are quantifiable and predictable, enabling qualitative inference of spatial and temporal differences in diet (e.g., Iverson et al. 1997, Käkelä et al. 2006).

Tufted puffins (*Fratercula cirrhata*) are considered the most pelagic of the Alcidae, spending the majority of the year distributed across the central North Pacific foraging primarily on invertebrates and forage fishes (Springer et al. 1999, Piatt & Kitaysky 2002). During the breeding season they require terrestrial nesting habitat and nest colonially in single-pair burrows located on small offshore rocks and islands. A single egg, laid between late May and early June, is incubated by both parents for about 45 days; hatching occurs mid to late July and chick fledging occurs from late August to early September. In this study, we used stable isotopes and FA signatures to infer seasonal dynamics of diets of tufted puffins breeding on a small island located on the northeast side of Kodiak Island from 2003-2005. We combined these molecular approaches with a more conventional technique for diet determination, collection of prey samples fed to nestlings obtained using the burrow screening method (Hatch & Sanger 1992). Our primary objectives were to determine whether diets of tufted puffins shift

across the breeding season and whether adults consume the same diet as nestlings. Because stable isotopes are potentially affected by physiological processes associated with nutritional state (Hobson et al. 1993, Cherel et al. 2005a), we also examined whether individual differences in stable isotope concentrations could be attributed to differences in body condition. We predicted whole blood from adults would become enriched in ^{15}N and ^{13}C as the breeding season progressed due to a shift from a pelagic distribution with a plantivorous diet during the non-breeding season to a near-shore distribution with a diet comprised of increasing amounts of fish during the breeding season. We also predicted chick-rearing adults would have lower $\delta^{15}N$ values than nestlings, indicating opportunistic consumption of small lower trophic level invertebrates that cannot profitably be delivered to nestlings. We investigated annual variation in carbon and nitrogen stable isotopes of potential prey items to establish whether annual differences in the isotopic composition of blood in puffins reflected shifts in diet or shifts in prey isotopes. Lastly, we used FA signature analysis to confirm differences detected using nitrogen and carbon stable isotopes as well as to infer species-level differences in diet that could not be elucidated from stable isotopes alone.

3.3 Methods

3.3.1 Study site and tissue sampling

Our study was carried out on Chiniak Island in Chiniak Bay on the northeast side of Kodiak Island, Alaska (57°40'N, 152°20W) during the breeding seasons (May through Aug) of 2003-05. Chiniak Island is approximately circular with a diameter of 0.5km and has a colony of >5000 breeding pairs of tufted puffins nesting in earthen burrows on grassy slopes along the perimeter of the island.

Adult tufted puffins were captured by reaching in through burrow entrances or by using a 7x10 m net draped over a cluster of 20-30 burrow entrances. We restricted our capture efforts to three distinct time periods: prior to egg-laying (22 May-2 June), late incubation (1-11 July), and late chick-rearing (23 Aug.-4 Sept.). During late chick-rearing, the majority of adults were captured after they flew in with bill loads to feed their

young. Nestlings were captured during the same time period as chick-rearing adults. We measured bill length (BL) and straight tarsus length (TL) using dial calipers (± 0.1 mm) and wing chord length (WCL; carpal joint to wing tip) using a ruler (± 1 mm). Birds were weighed with a spring scale (± 2 g).

We used a live biopsy technique, as described by Iverson et al. (2007), to remove approximately 100mg of adipose tissue for fatty acid signature analysis. Samples of adipose tissue were immediately placed in chloroform containing BHT (an antioxidant) and stored on ice until frozen. We collected blood samples for stable isotope analysis and molecular sexing by puncture of the alar or tarsal vein with a 23-guage needle. Blood was collected in heparinized 250 µl Natelson blood collecting tubes and transferred into 1.5 ml microcentrifuge tubes which were stored on ice until frozen. DNA was extracted from blood samples using a DNeasy tissue kit (QIAGEN Inc., Valentia, CA) and molecular sexing was performed according to the methods of Griffiths et al. (1998). We did not collect blood samples from adult puffins captured prior to egg-laying or during late incubation in 2003 and we did not determine nestling sexes.

3.3.2 Nestling diets

We collected chick meals by screening burrows either individually using small wire screens (15x15 cm) or in mass (20-30 burrows at a time) with a 7x10 m net. Either method effectively prevents adults from entering with food loads. Often these adults drop their bill loads at burrow entrances. A bill load sample is defined as all prey items collected from one burrow on one sampling date, but is not necessarily one bill load (bill load samples may be comprised of partial bill loads or multiple bill loads). Prey samples were later identified to species (where possible), weighed (± 0.1 g), and measured (± 0.1 mm). Samples were frozen and a subset was later analyzed for stable isotopes of nitrogen and carbon.

3.3.3 Stable isotope analysis

Samples of squid (*Berryteuthis magister* and *Gonatus kamtschaticus*) and euphausiids (adult female *Thysanoessa spinifera*) were obtained using mid-water trawls and ring nets during the summer months on the NE side of Kodiak Island. Euphausiid samples (2004: n = 6; 2005 n = 8) were from a single tow in each year whereas squid (2005 only: n = 10) were all captured in separate trawls. All other prey analyzed for stable isotopes were obtained from burrow screening (see Table 1 for sample sizes). Prey items and samples of whole blood were freeze dried and powdered using a mortar and pestle. The isotopic composition of protein in predator tissues generally reflects the composition of dietary protein (Gannes et al. 1998). Lipids are depleted in ^{13}C (DeNiro & Epstein 1977); therefore, they were extracted from prey items using a Soxtherm apparatus (Gerhardt, Bonn, Germany) with chloroform solvent. Samples were then dried at 60°C for 24 h to remove any residual solvent (Hobson et al. 1994). Because lipid extraction can affect $\delta^{15}N$ values (Sotiropolous et al. 2004), we also ran non-extracted samples. Non-extracted samples of euphausiids from 2004, salmonids (*Oncorhynchus spp.)* from 2005, and Pacific sandfish (*Trichodon trichodon*) from 2004 and 2005 were not available. We report $\delta^{15}N$ values of lipid-extracted prey in these instances because extraction did not affect $\delta^{15}N$ values of any of theses species in other years. Lipids were not extracted from whole blood because most of the organic material is contained in blood cells and $\delta^{13}C$ values are only slightly affected by the relatively small amount of lipid found in plasma (Cherel et al. 2005b). All samples were analyzed using a Costech Elemental Analyzer (ESC 4010), and Finnigan MAT Conflo III interface with a Delta+XP Mass Spectrometer. Replicate measurements of internal laboratory standards (peptone) indicated measurement error to be ± 0.3 ‰ for N and ± 0.1 ‰ for C. Stable isotope concentrations are reported using 'δ' notation according to $\delta X = [(R_{sample}/R_{standard})-1] \times 1000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Standard values are based on atmospheric N_2 for $\delta^{15}N$, and the Vienna Peedee Belemnite (VPDB) for $\delta^{13}C$.

3.3.4 Fatty acid signature analysis

We extracted lipids from adipose tissue samples according to Folch et al. (1957) as modified by Iverson et al. (2001). FA methyl esters (FAMEs) were prepared from ≤100 mg of the lipid extracts using 3.0 ml Hilditch Reagent (0.5 N H_2SO_4 in methanol) in 1.5 ml methylene chloride with BHT, capped under nitrogen and heated at 100°C for one hour (Budge et al. 2006). Following transesterification, FAMEs were extracted into hexane, concentrated using nitrogen gas, and then brought up to a final volume of 50 mg FAME/ml hexane. Identification and quantification of FAMEs was performed in duplicate using temperature programmed gas-liquid chromatography on a Perkin Elmer Autosystem II Capillary FID gas chromatograph fitted with a 30m x 0.25 mm column coated with (50% cyanopropyl) - methylpolysiloxane (DB-23) and linked to a computerized integration system (Tubochrom 4 software, PE Nelson, San Jose, CA). Each chromatogram was manually assessed for correct peak identification and reintegrated, where necessary.

3.3.5 Statistical analyses

Nestling Diets - All statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). Individual prey items within a burrow sample were not independent, nor were burrow samples collected on a given day. Therefore, we calculated a daily mean proportion for each prey type and used this daily mean in statistical analyses. Capelin (*Mallotus villosus*) and Pacific sandlance (*Ammodytes hexapterus*) were the two dominant species found in diets, with presence of one of these species generally reflecting absence of the other. Therefore, we investigated annual differences in diet by testing for differences in proportion of capelin using one-way ANOVA followed by post-hoc Tukey HSD tests for multiple comparisons among the three years.

Stable isotopes - We used general linear models to evaluate adult whole blood $\delta^{15}N$ and $\delta^{13}C$ (separately) during the breeding season using 16 candidate models selected *a priori* involving combinations of the following parameters: year, reproductive stage,

sex, body condition index (BCI), and interactions between year and reproductive stage. We included BCI in select models to determine whether stable isotope values were affected by nutritional stress, rather than simply reflecting shifts in diet. Residuals of mass regressed against PC1 from a principal component analysis on the three morphometric measurements (WCL, BL, and TL) were used as a BCI (Green 2001, Schulte-Hostedde et al. 2005). We calculated BCI separately for each sex, because the relationship between body mass and structural size is sex-specific in this species (see details in Williams et al. 2007a). Chick-rearing adults captured in 2003 were excluded from this analysis because isotopic data on birds captured prior to egg-laying and during late incubation was lacking. We used an information theoretic approach to model selection (Burnham & Anderson 2002) using Akaike's Information Criteria adjusted for small sample bias (AIC_c) and AIC_c "weights", w_i, the probability that a candidate model is the "best" given the data and the suite of models evaluated.

We examined the effects of age (adult vs. nestling) and year on $\delta^{15}N$ and $\delta^{13}C$ (analyzed separately) using two-way ANOVAs that included an age x year interaction term. The dataset used in this analysis consisted of adults captured during chick-rearing and nestlings in all years.

Fatty Acid Signatures - The number of FAs routinely identified in puffin adipose tissue (68) greatly exceeded the number of individuals in all of our sampling groups (n = 15-26). Thus, we selected the 14 FAs with the largest overall variances and overall means (≥0.9% of total FAs), excluding 22:5n-3, because it may be an intermediate of 20:5n-3 and 22:6n-3 (Ackman et al. 1988). These 14 FAs accounted for 89% by mass of the total FAs. Percentages of the 14 FAs were renormalized over 100% and then transformed into log ratios according to the following: $x_{trans} = \log (x_i/c_r)$ where x_i is the percentage of a given FA, x_{trans} is the transformed FA and c_r is 18:0, a reference FA (Budge et al. 2006). We transformed raw percentages into log ratios to break the constraint that each observation must sum to a constant (Aitchison 1986).

Differences in FA signatures among reproductive stages, years, and ages (nestling vs. adult) were evaluated using discriminant function analysis (DFA) followed by

classification using a jack-knifing procedure (leave-one-out cross-validation). Linear DFA assumes homogeneous covariance matrices between groups and uses a pooled covariance matrix for the construction of discriminant functions. The covariance matrices in our dataset were not homogeneous (Bartlett's test, $p < 0.001$) which may result in poorer separation between groups, although there is little evidence that moderate violation significantly alters cross-validated classification success (McGarigal et al. 2000). Quadratic DFA accounts for the heterogeneity of covariance matrices by using within-group covariance matrices in the construction of discriminant functions. However, sample sizes for each group ($n = 15$-26) in our dataset were small relative to the number of parameters used in DFA. This may result in poor estimation of within-group covariance matrices which can lead to poor classification success. Therefore, we report results of both linear and quadratic DFA. Linear and quadratic DFA were first performed separately for each year to determine cross-validated classification success among reproductive stages and ages and then were performed on the entire dataset to examine classification success among years. We also plot linear discriminant functions to elucidate the relationship among groups.

3.4 Results

3.4.1 Nestling diets

We collected 190, 150, and 158 bill load samples from burrow entrances comprised of 562, 714, and 375 prey items in 2003, 2004, and 2005, respectively. Nestling diets were comprised almost exclusively (>99.9%) of fish, primarily capelin, Pacific sandlance, and Pacific sandfish. A complete list of species collected by screening burrows is shown in Table 3.1. The proportion of capelin in bill loads differed significantly among years ($F_{2,21} = 15.24$, $p < 0.001$). Post-hoc analyses revealed bill loads contained significantly less capelin by mass in 2004 than in 2003 or 2005 (Tukey's HSD test, $p < 0.05$; Fig. 3.1).

3.4.2 Stable isotopes

During 2004 and 2005, whole blood $\delta^{15}N$ values of adult tufted puffins increased across the breeding season (Fig. 3.2a). The difference in mean $\delta^{15}N$ values between chick-rearing birds and pre-laying birds was 1.8 and 2.3 ‰ in 2004 and 2005, respectively. Assuming a step-wise increase of 3.4-3.8 ‰ per trophic level (TL; Minigawa & Wada 1984, Hobson & Welch 1992), we estimated the trophic shift from pre-lay to late incubation to be 0.47-0.53 TL and 0.61-0.68 TL in 2004 and 2005, respectively. Only 3 of the 16 candidate models for $\delta^{15}N$ were included in the 90% confidence set (Table 3.2). The best model included reproductive stage, year, and a stage x year interaction. The second and third best models added BCI and sex to the best model. However, inclusion of either of these additional parameters had little effect on the deviance and resulted in an increase in ΔAIC_c of >2, indicating they did little to improve model fit. Furthermore, the 95% confidence interval of parameter estimates for BCI [95% CI: -0.0020, 0.0014] and sex [-1.38, 1.37] bounded zero in these more complex models, suggesting no support for addition of either parameter. Thus, we concluded that only the model with reproductive stage, year, and a stage x year interaction was well supported by our data.

Whole blood $\delta^{13}C$ values changed with reproductive stage in both 2004 and 2005, but the shift was not consistent between years (Fig. 3.2b). Values of $\delta^{13}C$ tended to be lower prior to egg-laying compared to other stages of reproduction. Similar to $\delta^{15}N$, only 3 of the 16 candidate models for $\delta^{13}C$ were included in the 90% confidence set (Table 3.3). The best model included reproductive stage, year, and an interaction between reproductive stage and year. Addition of BCI or sex to the best model had little effect on the deviance and resulted in a jump in ΔAIC_c values of ~2, indicating no evidence to support inclusion of these parameters. Once again, the 95% confidence intervals of parameter estimates for BCI [-.0009, 0.0023] and sex [-0.09, 0.17] bounded zero. Thus, the best model based on ΔAIC_c was also the only model well supported by our dataset.

Based on $\delta^{15}N$, we found no evidence that chick-rearing adults feed at a lower trophic level than the nestlings they provision; adult blood was slightly enriched in ^{15}N compared to nestlings in 2003 (+ 0.12 ‰) and 2005 (+ 0.35 ‰), but slightly depleted in 2004 (- 0.32 ‰; Fig. 3.2a). Values of $\delta^{15}N$ in whole blood were significantly affected by year ($F_{2,99} = 6.509$, $p < 0.0001$), but not by age ($F_{1,99} = 0.006$, $p = 0.8$). However, there was a significant year x age interaction ($F_{2,99} = 1.77$, $p < 0.0003$). Differences between adults and nestlings in whole blood $\delta^{13}C$ were also inconsistent between years; compared to nestlings, chick-rearing adults were slightly enriched in ^{13}C in 2003 (+ 0.08 ‰) and 2005 (+ 0.17 ‰), but slightly depleted in 2004 (- 0.53 ‰; Fig. 3.2b). Similar to $\delta^{15}N$, $\delta^{13}C$ was significantly affected by year ($F_{2,99} = 49.539$, $p < 0.0001$). The effect of age was not significant ($F_{1,99} = 0.190$, $p = 0.2$), however, there was an interaction between age and year ($F_{2,99} = 2.442$, $p < 0.0001$).

Values of $\delta^{15}N$ and $\delta^{13}C$ varied among prey species and years (Fig. 3.3). Adult euphausiids were generally depleted in ^{15}N (indicative of a lower trophic level) and ^{13}C (indicative of a pelagic food web) compared to forage fishes, with the exception of capelin in 2005. As expected, annual differences in $\delta^{13}C$ values of forage fishes between years were consistent with the isotopic shift in nestling whole blood.

3.4.3 Fatty acid signatures

The FA signature of adipose tissue varied substantially between adults and nestlings as well as across reproductive stages (Table 3.4). Examination of within-group covariance matrices (not shown) suggested lack of homogeneity among groups was due to greater variance in FA signatures of adults captured prior to egg-laying and smaller variance in signatures obtained for nestlings (see FA SD reported in Table 3.4). Linear DFA within each year clearly showed differences by age and by reproductive stage (Fig. 3.4). The first linear discriminant functions accounted for 71, 45, and 74% of the total variation in 2003, 2004, and 2005, respectively. The second linear discriminant functions accounted for 24, 38, and 19% of the total variation in 2003, 2004, and 2005,

respectively. Following linear DFA, classification using a jackknife approach (leave-one-out cross-validation) resulted in 93, 99, and 98% of the observations being assigned to the correct group in 2003, 2004, and 2005. Quadratic DFA resulted in cross-validated classification success of 80, 92, and 88% in 2003, 2004, and 2005, respectively. The lower classification success in 2003 was due primarily to incorrect classification of pre-lay adults with only 40% (6 of 15) of individuals assigned to the correct group; 40% were incorrectly classified as incubating adults and 20% as chick-rearing adults.

When linear DFA was performed on the entire FA dataset, cross-validated classification success remained high; 90% were assigned correctly with 14 out of the 25 (56%) misclassified observations being assigned to the correct group but incorrect year. The first and second discriminant functions accounted for 47 and 16% of the total variation, respectively. The plot of group centroids for these two discriminant functions showed that differences between groups were generally much larger than differences between years within the same group (Fig. 3.5). The first and second discriminant functions showed that chick-rearing adults were generally closer to nestlings than to other adult reproductive stages in 2003 and 2005, but not in 2004. Cross-validated classification success following quadratic DFA was also quite high; 76% were assigned correctly. Classification success was >66% for all groups with the exception of pre-lay adults from 2003 from which all individuals (n = 15) were incorrectly classified; 13 of 15 (93%) individuals from this group were assigned to the correct stage of breeding but incorrect year. Sample size of pre-lay adults in 2003 (n = 15) was lower than any other period (n = 20-26).

3.5 Discussion

We found a consistent pattern of ^{15}N enrichment in whole blood of adult tufted puffins during the course of the breeding season, indicative of a 0.47 - 0.68 increase in trophic level of feeding from pre-lay to late chick-rearing. FA signatures differed among reproductive stages, as well as between chick-rearing adults and nestlings. With the exception of chick-rearing adults in 2004, annual variability in FA signatures appeared to

be much smaller than seasonal variability. Based on stable isotopes and FA signatures, we infer that trophic niche of adult tufted puffins is stage-dependent and that chick-rearing adults do not forage at a lower trophic level than nestlings but likely consume a different assortment of prey species.

3.5.1 Seasonal patterns

Shifts in foraging niches associated with reproductive stage may be indicative of an intrinsic shift in foraging behavior (i.e. stage-dependent foraging strategies) or a seasonal shift in prey availability (i.e. an extrinsic factor). Stable isotopes and FA signatures of adult puffins during pre-lay likely reflect spring diets when birds are distributed on their "wintering" areas. Values of $\delta^{15}N$ for pre-lay birds were consistent with stomach content analysis and indicated that tufted puffins fed primarily on lower-trophic level invertebrates during winter, consistent with previous studies (reviewed in Piatt & Kitaysky 2002). The requirement of terrestrial nesting habitat limits the distribution of puffins to within 100km of the colony during the breeding season (Piatt & Kitaysky 2002) and consequently their access to suitable foraging habitat is constrained. Thus, we suggest that an intrinsic factor, migration from pelagic wintering grounds to near-shore breeding grounds, is responsible for the shift in trophic level of feeding from pre-lay to late-incubation. However, we recognize that breeding in seabirds coincides with a seasonal increase in the abundance of forage fish in near shore waters (e.g., Blackburn & Anderson 1997), and intrinsic and extrinsic factors are therefore confounded. Stomach content analysis of puffins collected in the near-shore waters of Kodiak Island during breeding suggest birds consume predominantly fish during this period (Baird 1991), which is also consistent with information furnished from stable isotopes in our study. Nevertheless, $\delta^{15}N$ values of squid and adult euphausiids were close to values obtained for forage fishes and we cannot rule them out as important components of puffin diets during the breeding season.

Intermediate levels of $\delta^{15}N$ in incubating adult puffins may reflect feeding at an intermediate trophic level or incomplete turnover of nitrogen isotopes in blood from the

pre-lay period. The allometric equation for carbon turnover in blood developed by Carleton & Martinez del Rio (2005) provides an estimated half-life of 23.5 days for adult tufted puffins. Assuming a similar rate of turnover for nitrogen, as has been found in other birds fed high protein diets (Bearhop et al. 2002, Evans-Ogden et al. 2004), we estimate ~70% of nitrogen was replaced during the ~40 day interval between capture of pre-lay and incubating adults. Had incubating adults been feeding at the same trophic level as chick-rearing adults, 70% turnover would result in predicted $\delta^{15}N$ values of 13.4 and 14.2‰ in 2004 and 2005, respectively. Measured $\delta^{15}N$ values were lower than this in both years: 12.9‰ in 2004 and 13.9‰ in 2005. Thus, incomplete turnover of blood cells likely explains some, but not all, of the observed differences in $\delta^{15}N$ between incubating and chick-rearing birds. Values of whole blood $\delta^{13}C$ and adipose tissue FA signatures are also consistent with differences in foraging niches between incubating and chick-rearing adults.

Shifts in adult diet between incubation and chick-rearing may result from stage-dependent foraging strategies of seabirds. Breeding birds face time constraints that limit the duration and distance over which they may forage from a central place. Schaffer et al. (2003) found that incubating wandering albatross (*Diomedea exulans*) traveled 3.7 times farther and were at sea 3.2 times longer than chick-rearing birds. Estimates of median foraging ranges for common murres (*Uria aalge*), a short-range forager, were also much longer during incubation (37.8 km) compared to chick-rearing (5.4 km; Cairns et al. 1987). However, Piatt (1990) determined that common murres alter their foraging range in response to the influx of capelin into near-shore waters, illustrating the difficulty in disentangling effects of extrinsic and intrinsic factors on foraging behavior in seabirds. Charrassin et al. (1998) determined that chick-rearing king penguins (*Aptenodytes patagonicus*) dove deeper than incubating conspecifics during the same time periods; however, it is possible this variation is attributable to differences in bird quality. Humphreys et al. (2006) experimentally prolonged incubation in black-legged kittiwakes (*Rissa tridactyla*), thereby nullifying extrinsic effects, and found that chick-rearing birds

increased the frequency and duration of foraging bouts but did not alter their foraging distribution. We suggest that time budgets and/or foraging distribution of adult tufted puffins are likely to be stage-dependent and this is responsible for seasonal shifts in trophic niche. However, the possibility that predictable seasonal shifts in prey availability are responsible for the observed pattern cannot be discounted.

Prior studies provide some evidence that changes in seabird diets coincide with shifts in stage of reproduction. Ojowski et al. (2001) found that both time-budgets and diet composition differed between incubating and chick-rearing northern fulmars (*Fulmarus glacialis*). Breeding Wilson's storm petrels (*Oceanites oceanicus*) undergo a seasonal shift in diet coincident with the shift from incubation to chick-rearing, whereas non-breeders have no obvious change in diet during this time frame (Quillfeldt 2002). Baird (1991) also found differences in diet composition between breeding and non-breeding tufted puffins, supporting the premise that reproduction constrains diet selection in this species.

3.5.2 Adult vs. nestling diets

During the chick-rearing period, adults must locate suitable forage to provision their nestlings and to feed themselves. Based on central-place foraging theory, we predicted tufted puffins would select relatively large packages of high quality prey items to feed their nestlings. Consistent with this hypothesis, we found that tufted puffins fed their nestlings a diet comprised almost exclusively of fish. However, we found only small and inconsistent differences in $\delta^{15}N$ values between chick-rearing adults and nestlings, indicating adults were not self-feeding at a lower trophic level than nestlings.

It is possible that diets of nestlings and chick-rearing adults do not differ in any way. However, there are several other possible explanations for the observed similarity in the isotopic signatures of chick-rearing adults and nestlings. Given that we found no effect of prey size on stable isotope signatures within any of the species of forage fish we sampled (data not shown), adults could have consumed smaller prey but of the same species as was fed to nestlings and thus would not exhibit a consequent effect on blood

$\delta^{15}N$ values. Alternatively, adults could have been consuming significant amounts of squid and adult euphausiids and, since squid and euphausiid $\delta^{15}N$ values differed little from forage fishes, this dietary influence would have had little effect on adult isotope signatures.

Addition of FA signature analysis helps in discriminating diets of nestlings and adults. FA signatures suggested adult diets differed from nestlings, particularly in 2004. Incomplete turnover of FAs in adult puffins is not likely a factor because chick-rearing adults did not have intermediate FA signatures between incubating adults and nestlings (see Table 3.4). Although, $\delta^{15}N$ values indicate nestlings are not fed higher trophic level prey than breeding adults consume, it is possible that they are fed higher quality prey. For example, Wilson et al. (2004) determined that breeding common murres feed their young a higher quality diet compared to what they consume for self-maintenance. Romano et al. (2006) demonstrated growth rates of tufted puffin nestlings fed low quality prey were considerably lower than nestlings fed the same biomass of high quality prey. Although growth rates of tufted puffin chicks fed high quality vs. low quality diets are similar when diets are iso-caloric (Romano et al. 2006), the energetic costs associated with delivering more and/or heavier bill-loads to compensate for low quality are likely to be high.

3.5.3 Effects of nutritional stress

Nutritional stress is thought to cause ^{15}N enrichment of animal tissues due to catabolism of endogenous protein stores, recycling of metabolic amino acids, and discrimination against the heavier isotope during formation of nitrogenous wastes (Macko et al. 1986, Hobson et al. 1993, Gannes et al. 1998). Although this hypothesis has been well validated in studies of fasting animals (Hobson et al. 1993, Cherel et al. 2005a), studies of moderately restricted growing birds have found either no effect (Kempster et al. 2007) or opposing effects (Williams et al. 2007b). Nevertheless, nutritional stress is sometimes invoked as a cause of $\delta^{15}N$ enrichment in field studies without this hypothesis being tested directly (e.g. Baduini et al. 2006). We found no

support for an effect of body condition index on either $\delta^{15}N$ or $\delta^{13}C$ values in adult tufted puffins despite the fact that chick-rearing birds were in poorer body condition than incubating birds (Williams et al. 2007a) and chick-rearing birds had the highest $\delta^{15}N$ values. Our result is not surprising given that seasonal mass loss in the closely related rhinoceros auklet (*Cirorhinca monocerata*) is due almost exclusively to loss of lipid stores (Niizuma et al. 2002) and enrichment of ^{15}N in body tissues is only expected if poor body condition is associated with protein loss (Martinez Del Rio & Wolf 2005).

Seasonal mass loss in breeding seabirds may be regarded as a consequence of a predictable life-history event, rather than an artifact of stress. Loss of body mass during reproduction has been detected in numerous alcids including least auklets (*Aethia pusilla*; Jones 1994), Atlantic puffins (*Fratercula arctica*; Barrett & Rikardsen 1992), and thick-billed murres (*Uria lomvia*; Croll et al. 1991) and may be an intrinsic process that also functions to reduce wing-loading during the energetically demanding chick-rearing period (Norberg 1981). It is likely that severe nutritional stress, such as was observed for short-tailed shearwaters (*Puffinus tenuirostris*) in the study of Baduini et al. (2006), may result in ^{15}N enrichment of body tissues. However, we suggest the nutritional stress hypothesis be tested in future studies using a weight of evidence approach by including an index of body condition or a direct measure of energy stores as a covariate in models for nitrogen stable isotopes.

3.5.4 Conclusions

Although egg-laying phenology of puffins is correlated with oceanographic conditions (Durant et al. 2003, Gjerdrum et al. 2003), it is unclear which environmental cue birds respond to at this stage because their diets are poorly characterized in the early breeding season. Based on stable isotopes and FA signatures, we infer the foraging niche of adults changes over the course of the breeding season and we suggest these changes are due to transition from feeding in wintering areas as well as constraints imposed by stage of reproduction. Furthermore, FA signatures are consistent with dietary differences between chick-rearing adults and nestlings. Therefore, we suggest information furnished

from nestling diets should not be extrapolated to adults or to other time periods. Although puffin chick diets and growth rates are potentially useful as indicators of forage fish availability (Hatch & Sanger 1992, Barrett 2002), this only furnishes information for a relatively narrow time period each year. In order to utilize information furnished from reproductive parameters measured outside of the chick-rearing period, better knowledge of which aspect(s) of the environment adults are responding to (physiologically, behaviorally etc) and thus what they are indicating, is needed. In particular, species-level estimates of adult diets are necessary to better understand the relationship between tufted puffins and supporting food webs. Future studies attempting to disentangle extrinsic and intrinsic factors affecting seasonal changes in adult diets are also needed.

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Table 3.1 *Fratercula cirrhata*. Prey species obtained by screening tufted puffin burrows on Chiniak Island, Kodiak, AK from 2003-2005. Numbers of prey used in stable isotope analysis are shown in brackets.

		2003		2004		2005	
Common Name	Scientific Name	n (n_{SI}[a])	Mean Mass (SD)	n (n_{SI}[a])	Mean Mass (SD)	n (n_{SI}[a])	Mean Mass (SD)
Capelin	*Mallotus villosus*	177 (5/7)	5.1 (3.8)	85 (12/7)	6.5 (2.2)	272 (10/7)	4.1 (2.1)
Pacific Sandlance	*Ammodytes hexapterus*	366 (5/7)	2.1 (2.3)	564 (13/7)	1.8 (1.9)	74 (10/7)	4.6 (2.7)
Pacific Sandfish	*Trichodon trichodon*	18 (5/7)	6.6 (2.1)	20 (9)	4.2 (1.3)	12 (10)	4.9 (2.4)
Salmonid	*Oncorhynchus spp.*	0	.	6 (6)	14.4 (13.7)	8 (7/7)	13.3 (5.0)
Pacific Cod	*Gadus macrocephalus*	0	.	27 (6/7)	3.4 (2.8)	0	.
Walleye Pollock	*Theragra chalcogramma*	0	.	9	1.8 (0.5)	0	.
Soft Sculpin	*Psychrolutes sigalutes*	0	.	2	2.4 (0.7)	4	1.8 (0.5)
Prowfish		0	.	0	.	1	8.6
Snake Prickleback	*Lumpenus sagitta*	0	.	1	1.9	0	.
Flatfish	Unidentified	0	.	0	.	3	0.3 (0.0)
Squid	Unidentified	1	0.53	0	.	1	0.5
Shrimp	Unidentified	0	.	0	.	7	0.2 (0.0)

[a]Sample size for stable isotope analysis (# lipid extracted / # non-extracted)

Table 3.2 *Fratercula cirrhata.* A summary of the 90% confidence set (cumulative w_i = 0.90) of general linear models that best explain $\delta^{15}N$ of adult tufted puffins (n = 84) captured at Chinak Island in 2004-05. The model set is presented in descending order of w_i, and was extracted from an initial set of 16 candidate models selected *a priori.* Models incorporated parameters of reproductive stage (stage), year, body condition index (BCI), and sex.

Model	K[a]	Deviance	ΔAIC_c	w_i[b]
Stage, year, year x stage	7	-218.6	0.0	0.59
Stage, year, year x stage, BCI	8	-218.6	2.3	0.19
Stage, year, year x stage, sex	8	-218.6	2.5	0.17

[a]Number of parameters
[b]Model weight

Table 3.3 *Fratercula cirrhata.* A summary of the 90% confidence set (cumulative w_i = 0.90) of general linear models that best explain $\delta^{13}C$ of adult tufted puffins (n = 84) captured at Chinak Island in 2004-05. The model set is presented in descending order of w_i, and was extracted from an initial set of 16 candidate models selected *a priori.* Models incorporated parameters of reproductive stage (stage), year, body condition index (BCI), and sex.

Model	K[a]	Deviance	ΔAIC_c	w_i[b]
Stage, year, year x stage	7	-228.1	0.0	0.51
Stage, year, year x stage, BCI	8	-228.9	1.7	0.22
Stage, year, year x stage, sex	8	-228.6	2.0	0.19

[a]Number of parameters
[b]Model weight

Table 3.4 *Fratercula cirrhata*. Fatty acid composition of adipose tissue from tufted puffins captured in Chiniak Bay, Kodiak Is., AK. Values are mean (SD) weight percentage. Only the 14 (of 68) most abundant fatty acids are shown.

	Pre-lay Adults			Incubating Adults			Chick-rearing Adults			Nestlings		
	2003	2004	2005	2003	2004	2005	2003	2004	2005	2003	2004	2005
	n=15	n=22	n=23	n=20	n=21	n=22	n=22	n=26	n=21	n=23	n=23	n=22
14:0	4.1	4.1	4.2	4.7	3.6	4.8	4.4	3.0	5.1	5.0	4.5	5.8
	(0.7)	(1.0)	(0.8)	(1.0)	(0.9)	(0.6)	(0.8)	(0.5)	(1.1)	(0.5)	(0.6)	(0.4)
16:0	18.9	17.3	19.5	18.6	19.9	18.4	20.2	16.8	20.4	20.1	20.3	21.0
	(2.6)	(3.2)	(3.6)	(2.4)	(1.7)	(1.9)	(1.3)	(4.1)	(1.5)	(1.1)	(1.4)	(0.5)
16:1n-7	3.8	3.6	3.6	4.2	3.8	4.2	5.5	2.6	5.6	5.7	4.8	6.9
	(0.8)	(0.9)	(0.8)	(0.7)	(0.6)	(0.6)	(1.0)	(0.7)	(1.1)	(0.6)	(0.5)	(0.4)
18:0	4.4	4.4	5.0	5.9	6.9	5.2	6.9	9.3	5.9	5.7	5.6	6.9
	(1.1)	(1.0)	(1.4)	(1.3)	(1.4)	(0.9)	(1.5)	(1.3)	(1.7)	(1.1)	(0.7)	(0.4)
18:1n-9	16.8	14.4	16.5	14.0	13.8	13.2	15.4	11.2	15.2	16.4	16.4	16.4
	(2.4)	(4.1)	(2.6)	(1.8)	(1.9)	(1.3)	(1.6)	(3.2)	(2.0)	(1.2)	(1.9)	(0.9)
18:1n-7	4.0	3.0	3.8	2.5	2.1	2.2	2.7	2.2	2.5	3.2	2.3	3.3
	(0.9)	(1.1)	(1.1)	(0.4)	(0.3)	(0.3)	(0.5)	(0.4)	(0.3)	(0.2)	(0.2)	(0.1)
18:2n-6	0.7	0.8	0.8	0.9	0.8	1.0	1.3	1.1	1.3	1.1	1.3	1.6
	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.1)	(0.2)	(0.1)	(0.2)	(0.1)	(0.1)	(0.1)
18:4n-3	0.7	0.7	0.8	1.1	0.9	1.2	1.0	0.8	1.1	0.9	1.1	1.4
	(0.2)	(0.2)	(0.3)	(0.3)	(0.2)	(0.3)	(0.3)	(0.4)	(0.3)	(0.2)	(0.3)	(0.2)
20:1n-11	7.2	9.4	6.5	7.6	6.3	7.8	2.4	5.5	3.7	5.5	6.1	4.0
	(2.7)	(3.4)	(2.9)	(2.6)	(2.1)	(2.0)	(1.0)	(2.7)	(0.9)	(1.3)	(2.0)	(0.7)
20:1n-9	2.6	3.9	3.5	2.3	3.7	3.3	2.9	3.6	2.5	2.7	3.7	2.6
	(0.8)	(1.4)	(1.5)	(0.5)	(0.9)	(0.5)	(0.6)	1.0)	(0.4)	(0.2)	(0.6)	(0.2)
20:5n-3	9.3	7.6	7.6	8.2	8.1	6.6	8.8	6.6	8.3	7.0	5.4	7.0
	(2.3)	(2.9)	(3.0)	(2.3)	(1.2)	(1.9)	(1.8)	(2.0)	(1.4)	(0.8)	(1.1)	(1.1)
22:1n-11	10.9	13.8	12.8	9.3	9.0	13.0	5.1	8.6	6.8	6.1	7.8	5.5
	(4.0)	(5.8)	(5.4)	(3.6)	(2.3)	(3.7)	(1.7)	(3.7)	(2.2)	(1.3)	(2.4)	(0.9)
22:1n-9	1.1	1.2	1.1	0.7	0.7	0.9	0.7	0.8	0.7	0.5	0.6	0.5
	(0.4)	(0.4)	(0.4)	(0.2)	(0.2)	(0.2)	(0.2)	(0.3)	(0.2)	(0.1)	(0.1)	(0.1)
22:6n-3	6.6	6.9	5.7	9.1	10.2	7.9	10.4	9.4	9.4	9.1	8.7	7.9
	(1.1)	(2.5)	(1.9)	(1.8)	(1.5)	(1.6)	(1.4)	(2.8)	(1.3)	(1.0)	(1.7)	(1.1)

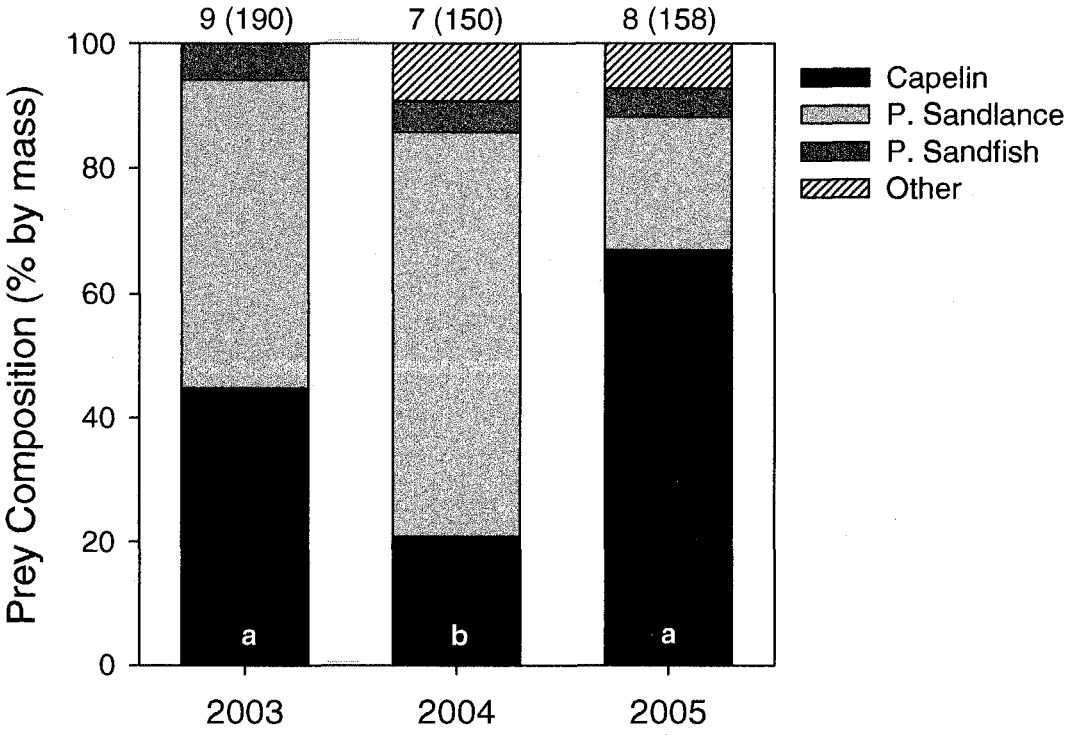

Fig. 3.1 *Fratercula cirrhata*. Percent composition by mass of tufted puffin nestling diets from 2003-05 on Chiniak Island as determined by burrow screening. The number of sampling days is shown above each bar with the total number of burrow samples collected in brackets. Different letters indicate significant differences (Tukey HSD test: $p < 0.05$) in the proportion of capelin in chick diets between years. Scientific names of forage fishes are shown in Table 3.1.

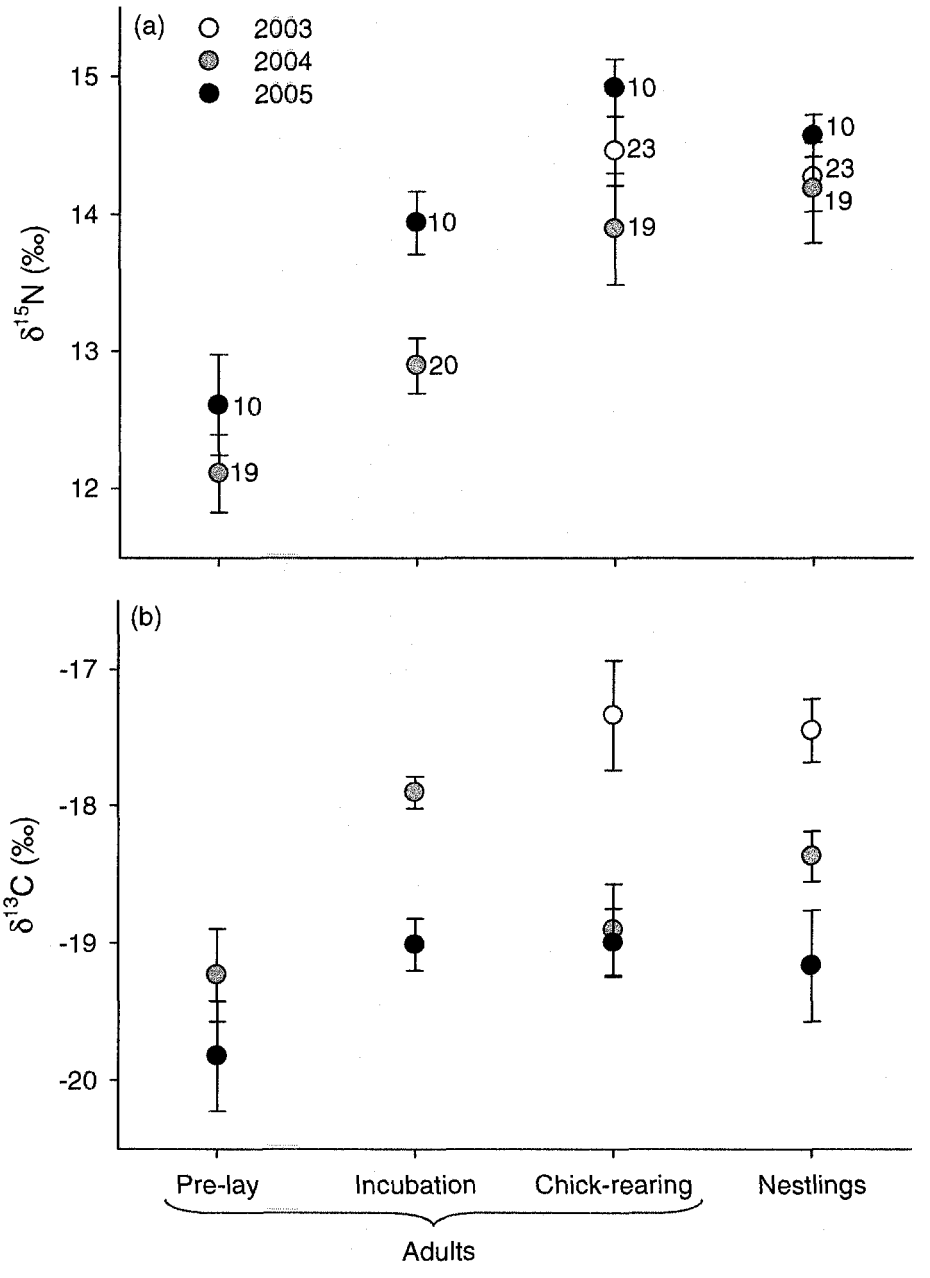

Fig. 3.2 *Fratercula cirrhata.* Values (mean ± SD) of (a) stable-nitrogen and (b) stable-carbon in whole blood of nestling and pre-lay, incubating, and chick-rearing adult tufted puffins captured on Chiniak Island in 2003-2005. Sample sizes are shown next to each mean value in panel (a).

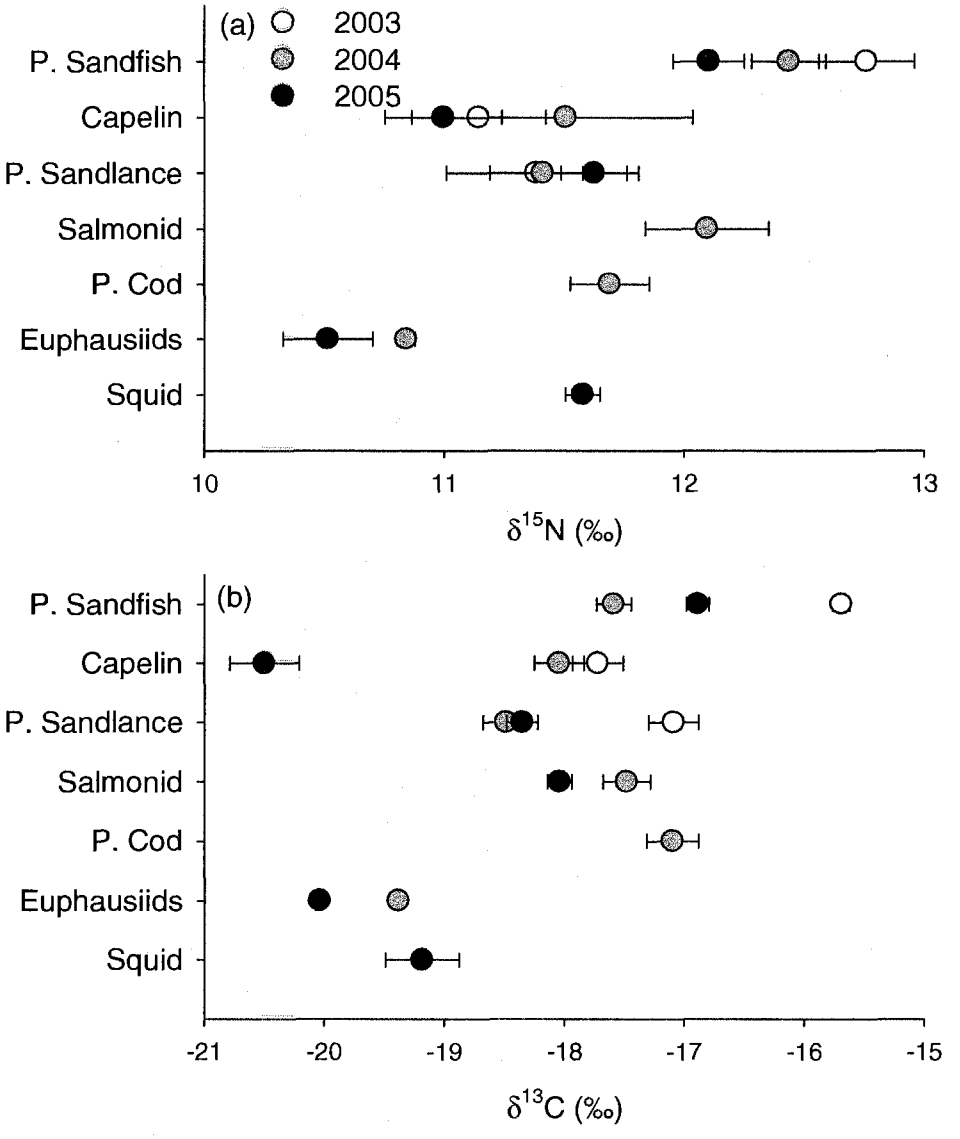

Fig. 3.3 *Fratercula cirrhata.* Values (mean ± SE) of (a) stable-nitrogen and (b) stable-carbon for forage fishes delivered to nestlings on Chiniak Island and for invertebrates (squid and euphausiids) collected in nets in near-shore waters northeast of Kodiak from 2003-2005. Scientific names and sample sizes are shown in Table 3.1.

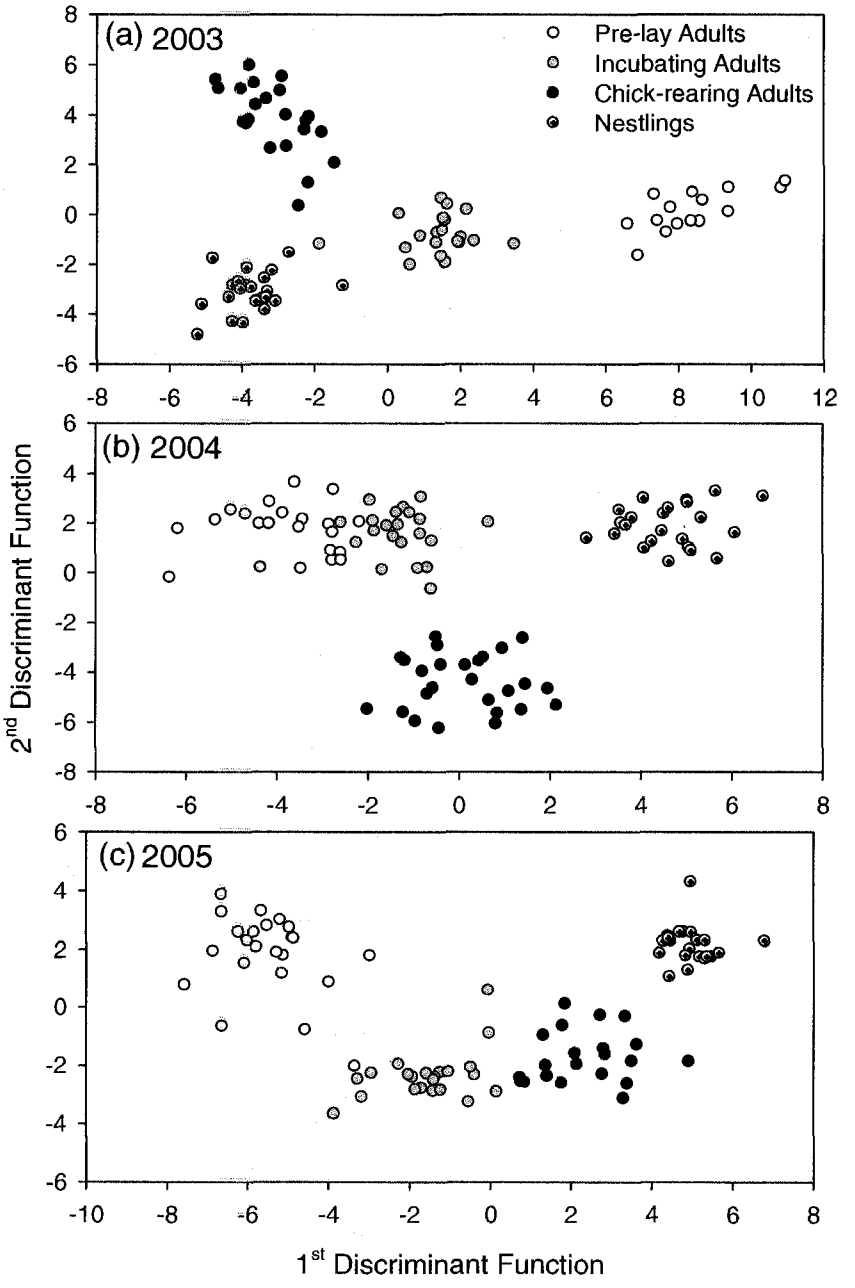

Fig. 3.4 *Fratercula cirrhata.* Discriminant scores from linear DFA based on fatty acid signatures of pre-lay, incubating, and chick-rearing adults and nestlings in (a) 2003, (b) 2004, and (c) 2005. Cross-validated classification success was 93, 99, and 98% in 2003, 2004, and 2005, respectively.

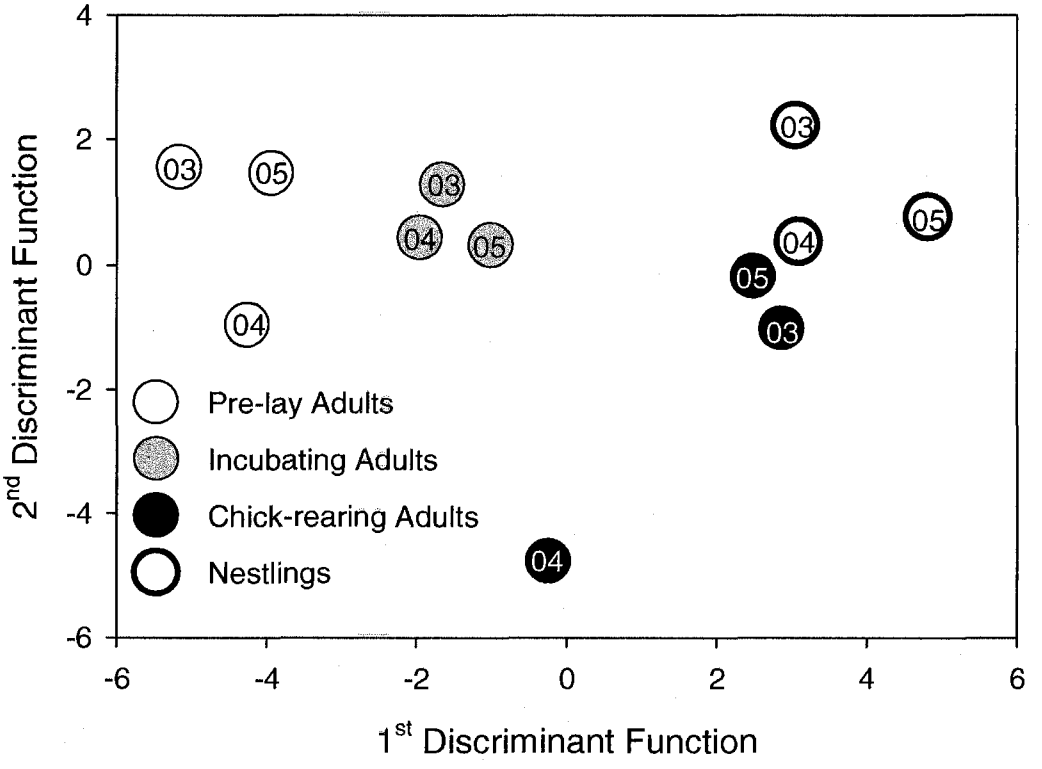

Fig. 3.5 *Fratercula cirrhata*. Group centroids from linear DFA on fatty acid signatures of pre-lay, incubating, and chick-rearing adult and nestling tufted puffins captured from 2003-05. Numbers within each symbol indicate year. The first and second discriminant function accounted for 47 and 16% of the total variation, respectively. Cross-validated classification success was 90% with 14 of 25 misclassified observations being classified to the correct group but wrong year. Sample sizes for each group are shown in Table 3.4.

Chapter 4: Sex-specific differences in body condition indices and seasonal mass loss in Tufted Puffins[1]

4.1 Abstract

Reduced prey availability can affect the growth and survival of nestling seabirds. However, few studies have demonstrated similar effects on indices of adult body condition. We examined body condition and seasonal mass loss of breeding adult male and female Tufted Puffins (*Fratercula cirrhata*) at Chiniak Bay, Kodiak Island, Alaska in 2004-2005. We determined sex using genetic analysis, developed a discriminant function to determine sex using morphometric measurements, and examined the body condition of adult males and females relative to the growth rates of their offspring. We found that morphological measurements were only moderately useful for sexing Tufted Puffins, with 74% of adults (N = 176) correctly classified. We also found that the relationship between adult body mass and size differed between sexes and conclude that body condition indices must be calculated separately for each sex to avoid inter- and intrasexual bias. Body condition of male and female Tufted Puffins declined during the chick-rearing period. However, body condition of females did not differ between years, whereas male condition declined to a greater degree during 2004 when the mass of young at fledging was significantly lower. Although these results suggest that adult male Tufted Puffins sacrifice their own body condition in years of diminished nestling growth and females do not, reasons for this apparent intersexual difference in reproductive strategies remain unclear.

[1]Previous version published as: Williams CT, Kildaw SD, Buck CL (2007) Sex-specific differences in body condition indices and seasonal mass loss in tufted puffins. Journal of Field Ornithology 78: 369-378

4.2 Introduction

Marine ecosystems are dynamic environments with oceanographic conditions fluctuating at a variety of temporal and spatial scales. Perturbations in physical state affect marine food webs (McGowan et al. 1998, Anderson and Piatt 1999) and, in turn, affect the reproductive success of seabirds by altering the availability of prey for young (Hedd et al. 2002, Durant et al. 2003) and by limiting the energy reserves of adults (Chastel et al. 1995). The effects of reduced prey availability on nestling growth and fledging success are well documented (Litzow et al. 2002, Suryan et al. 2002), but few studies have demonstrated similar effects on indices of adult body condition (Bolton et al. 1993, Kitaysky et al. 1999).

Life-history theory stipulates that animals face a tradeoff between current reproductive effort and future reproductive output (Lack 1968). Thus, a 'cost of reproduction' exists and animals must balance investment in current reproduction with their own probability of survival in a manner that maximizes lifetime fitness (Williams 1966). Seabird life-histories are characterized by relatively long lives with low annual fecundity and, therefore, even a small survival cost can have substantial ramifications for lifetime fitness.

Experimental manipulation of reproductive effort in seabirds has demonstrated a cost of reproduction in terms of survival (Golet et al. 1998) and future reproductive output (Wernham and Bryant 1998). However, it is less clear how costs are adjusted in response to reduced prey availability. Supplemental feeding experiments that manipulate nestling food demand have demonstrated that adults will reduce provisioning effort in response to decreased demand (Wernham and Bryant 1998, Harding et al. 2002, Gjerdrum 2004). Cross-fostering experiments have also shown that adults can modulate provisioning in response to chick needs (Bertram et al. 1996, Hamer et al. 1998, Varpe et al. 2004). However, studies seeking to determine if adult seabirds will compromise their own body condition to continue provisioning offspring when prey availability decreases or offspring demand increases have produced conflicting results (Barrett and Rikardsen 1992, Takahashi et al. 1999, Weimerskirch et al. 2001, Gaston and Hipfner 2006).

Moreover, sex-specific roles in parental care and differences in capacity to store energy reserves may lead to sex-specific modulation of body condition.

Three general scenarios can be envisioned for responses of chick-rearing seabirds to limited prey availability (Gaston and Hipfner 2006): 1) adults maintain body condition and modulate rates of chick-provisioning with food availability, 2) rates of nestling provisioning are held constant and adult body condition varies with food availability, and 3) adult body condition and chick-provisioning rates are simultaneously adjusted. It is unlikely that any of these scenarios operates across the entire spectrum of food availability; upper and lower thresholds for adult body condition likely exist and adults may compromise body condition to provision nestlings at some minimum rate. Additionally, body condition of adults and growth of nestlings are potentially buffered by flexible time budgets (Burger and Piatt 1990). Nevertheless, previous studies have detected correlations between body condition and food abundance (Bolton et al. 1993, Kitaysky et al. 1999), as well as simultaneous adjustment of adult body mass and provisioning effort (Weimerskirch et al. 2001, Gaston and Hipfner 2006). Thus, body condition of some species of seabirds may reflect variation in food availability during the breeding season.

Studies of the seasonal dynamics of body condition generally involve scaling body mass to structural size as an index of body condition because accurate measurement of body stores necessitates sacrificing the bird and requires time-intensive chemical analyses. Alternatively, seasonal dynamics of body condition can be examined using ANCOVA, with mass as the dependent variable, season as a factor, and an index of structural size as a covariate (Garcia-Berthou 2001). Both methods assume that mass scaled to structural size provides a useful estimate of body condition (defined as energy stores scaled to body size), but this has not been validated for Tufted Puffins (*Fratercula cirrhata*). However, Niizuma et al. (2002) found that differences in mass between incubating and chick-rearing Rhinoceros Auklets (*Cerorhinca monocerata*; actually a puffin species) were due primarily to loss of lipid reserves. Although indices of body condition are often calculated separately for males and females, sexual dimorphism is

subtle in many seabird species and, therefore, sexes are sometimes pooled for analyses (Chastel et al. 1995, Tveraa and Christensen 2002). Pooling sexes prior to analysis assumes accounting for structural size eliminates the effect of sex on body mass but will introduce bias if the relationship between body mass and structural size is sex-specific.

We examined the seasonal dynamics of adult body condition in Tufted Puffins and relate inter-annual differences in body condition to the growth rate and survival of nestlings. Our primary objective was to determine how adult male and female Tufted Puffins prioritize competing goals of maintaining their own body condition and maximizing the growth rate of their offspring. We developed a body condition index by first determining sex of adult Tufted Puffins using both molecular techniques and morphometric measurements, then determined whether the relationship between mass and structural size was sex-specific.

4.3 Methods

4.3.1 Study site

Our study was conducted during the breeding seasons of 2004-2005 on Chiniak Island within Chiniak Bay northeast of Kodiak Island, Alaska (57°40'N, 152°20W). Chiniak Island is approximately circular with a diameter of 0.5 km and has a colony of > 5000 breeding pairs of Tufted Puffins (CTW, pers. obs.) nesting in earthen burrows on grassy slopes around the perimeter of the island.

4.3.2 Chick growth and fledging mass

We began monitoring burrows on 24 July in 2004 and on 22 July in 2005. Nestlings that could not be reached through the entrance were accessed using holes excavated in previous years and sealed with either plywood or flat rocks. We weighed chicks using spring scales (± 2 g) and measured flattened wing length from the wrist to the wingtip (± 1mm) every 4-5 days throughout the nestling period and every 4 days as chicks approached fledging age. When hatch date was unknown, we estimated age using a wing length vs. age regression derived from known-age nestlings. We calculated growth rate

for each nestling as the slope of the linear regression equation relating mass and age between ages 10 and 30 days, the near-linear portion of the growth curve (Gjerdrum 2001). Nestlings with fewer than three measurements of mass between 10 and 30 days of age were excluded from growth rate analyses. We defined fledging success as the number of chicks reaching a minimum wing length of 130 mm per egg hatched (Gjerdrum et al. 2003) and assumed chicks that disappeared before attaining this minimum were dead. We defined the peak mass of an individual as the maximum mass measured during the nestling period and fledging mass as the final mass recorded prior to fledging (disappearance of nestlings with a wing length > 130 mm). Mass recession was defined as the difference between peak mass and fledging mass. Nestlings that had not fledged (1 of 35 in 2004 and 2 of 44 in 2005) by the day of the final nest check (12 Sept 2004 and 11 Sept 2005) were excluded from analysis of fledging mass.

4.3.3 Adult condition

Because reproductive success and growth rates of Tufted Puffin chicks can be influenced by investigator disturbance (Pierce and Simons 1986, Whidden et al. 2007), we captured all adults from burrows located outside of growth monitoring plots. Adult Tufted Puffins were captured by hand in their burrows or with a 7 x 10 m net draped over a cluster of 20-30 burrow entrances. If an adult was captured more than once, we included only data from the first capture in our analysis to ensure all observations were independent. We restricted capture efforts to four periods: prior to egg-laying (22 May-2 June), late incubation (1-11 July), early-chick rearing (2005 only; 4-13 August), and late chick-rearing (23 August-4 September). For each adult, we determined wing chord length (WCL; carpal joint to tip of longest primary), bill length (BL), and straight tarsus length (TL). Wing chord was measured using a ruler (± 1 mm), whereas bill and tarsus measurements were taken using dial calipers (± 0.1 mm). Body mass was determined using a spring scale (± 2 g). Blood samples were collected in heparinized 250 µl Natelson tubes after puncturing either the alar or tarsal vein with a 25-guage needle. Blood was transferred immediately into 1.5 ml microcentrifuge tubes and stored on ice until frozen

as either whole blood or blood cells at -20°C until analysis. DNA was extracted using a DNeasy tissue kit (QIAGEN Inc., Valentia, CA) and determination of sex was performed according to the methods of Griffiths et al. (1998).

4.3.4 Statistical analyses

We performed all statistical analyses using the Statistical Analysis System (SAS Institute 2006) with the α-level set at 0.05. With the exception of nestling mass recession and adult female tarsus lengths, all data passed assumptions required for parametric statistical tests (Sokal and Rohlf 1981). We used a non-parametric test (Mann-Whitney *U* test) to compare nestling mass recession between years. Tarsus lengths of females were not normally distributed due to slight negative skew and mild leptokurtosis. A normal distribution of tarsus lengths was obtained using a doubly-reflected square-root transformation (3-sqrt(40.4-tarsus)). However, data transformation had no effect on results, including classification success based on discriminant function analysis. Therefore, we report analyses based on raw values to maximize the utility of the discriminant function in a field setting.

We compared growth rate of nestlings, peak mass, and mass at fledging between years using Student's t-tests with a Bonferroni correction for multiple comparisons. We tested for inter-annual differences in fledging success using Fisher's Exact Test. Student's t-tests with a Bonferronni correction were used to determine if morphometric parameters differed between the sexes. We assumed that sex determined by genetic analysis was correct and used discriminant function analysis (DFA) and classification using a jack-knifing procedure (cross-validation) to assess the accuracy of sexing via morphometric measurements. The linear discriminant function for each sex was reduced into a single discriminant function by subtracting the 'male' function from the 'female' function (Jodice et al. 2000). We developed a sexes-pooled body size index (Rising and Somers 1989, Green 2001) by performing a principal component analysis (PCA) on the three structural measurements (WCL, BL, TL) and extracting the first principal component (PC1).

We examined the seasonal dynamics of body condition index using ANCOVA (in GLM using Type III SS). Adults captured during early chick-rearing in 2005 (N = 13 females and 10 males) were excluded from this analysis because we did not capture any birds during this period in 2004. Three females had outlier PC1 scores, with two substantially smaller in all morphometric measures and the third the size of a large male. Inclusion of these three birds had no effect on results of statistical tests, but they did influence parameter estimates and, therefore, we excluded them from all analyses. Body mass was the dependent variable in our model, whereas sex, reproductive stage, and year were included as factors along with body size index (PC1); a covariate that controls for structural size (Garcia-Berthou 2001). The initial global model included all two-way interactions. Interaction terms were subsequently dropped when $P > 0.10$. Simple effects tests (LSMEANS/SLICE) were used to examine significant two-way interactions A x B (i.e. year x reproductive stage). This procedure tests for effects of A for each B, which is calculated by extracting the appropriate row from the coefficient matrix for the A x B LSMEANS and using it to form an F-test. When interactions were not significant, we tested for differences between reproductive stages using Tukey's HSD test to control for multiple comparisons. Sex-specific differences in regulation of body condition in response to experimental manipulation of reproductive effort have previously been reported for seabirds (Velando and Alonso-Alvarez 2003). Therefore, we decided *a priori* to repeat the analysis separately for male and female puffins. For this analysis, we developed sex-specific body size indices in the same manner as for the sexes-pooled dataset by extracting PC1 from a PCA on the three structural measurements.

Residuals of mass regressed against PC1 from a PCA on morphometric measures are commonly used as an index of body condition (see references in Green 2001 and Schulte-Hostedde et al. 2005). Therefore, we applied this technique to the pooled-sexes dataset. We then regressed the output residuals (sexes-pooled residuals) against the sexes-pooled body size index separately for each sex to determine if the sex-specific relationship between body mass and structural size detected in our original ANCOVA produced intra-sexual bias in indices of body condition. Finally, to determine if residuals

were still affected by body size index when controlling for other effects, we ran separate ANCOVAs for each sex with the sexes-pooled residual as the dependent variable and reproductive stage, year, and the year*stage interaction as factors, and with the sexes-pooled body size index as a covariate. Values are provided as mean ± 1 SE unless noted otherwise.

4.4 Results

4.4.1 Nestling growth and fledging mass

For both years, the estimated mean hatch date was 20 July and mean fledging date was 3 September. Fledging success did not differ between years (Fisher's Exact Test: P = 0.40), with success rates of 87.9% (29 of 33 hatchlings) in 2004 and 95.2% (40 of 42) in 2005. Growth rates of puffin chicks were significantly lower in 2004 than 2005 (14.35 ± 0.47 g/day and 16.18 ± 0.57 g/day, respectively; t=-2.5, P=0.017, N= 28 and 41, respectively). The difference in mean growth rate between years was 1.9 g/day (95% CI: 0.3, 3.3). Peak mass of nestlings was also significantly lower in 2004 compared to 2005 (526.1 ± 10.0 g and 583.5 ± 11.9 g, respectively; t = -4.8, $P < 0.0001$, N = 28 and 38, respectively). The difference in mean peak mass between years was 57.4 g (95% CI: 33.7, 81.1). Mass at fledging was significantly lower in 2004 than 2005 (467.3 ± 11.9 g and 563.6 ± 8.2 g, respectively; t = -6.87, $P < 0.0001$, N = 28 and 38, respectively). The difference in mean fledging mass between years was 96.3g (95% CI: 66.3, 122.0). Mass recession was significantly greater in 2004 (58.8 ± 8.8 g, N = 28) than 2005 (19.9 ± 5.4 g, N = 38; U = 12.3, P = 0.001). Thus, reduced fledging mass in 2004 was due to a combination of lower peak mass and greater mass recession (Fig. 4.1).

4.4.2 Sex determination

DNA analysis of 176 adult Tufted Puffins revealed 69 males and 107 females. Males had larger tarsus (t = -5.7, $P < 0.0001$), wing chord (t = -4.0, $P < 0.0001$), and culmen (t = -6.1, $P < 0.0001$) measurements than females. Adult male and female Tufted Puffins had mean tarsus lengths of 36.3 ± 1.2 (SD) mm and 35.3 ± 1.2 mm, mean wing

chord lengths of 204.0 ± 4.5 mm and 201.2 ± 5.0 mm, and mean culmen lengths of 60.5 ± 2.4 mm and 58.4 ± 2.1 mm, respectively. The first discriminant function (D1) from a DFA classified 130 of 176 (74%) observations to the correct group: D1= 57 – (0.53878 x TL) – (0.35291 x BL) – (0.07977 x WCL). Adults with greater discriminant scores than the cutoff (-0.025) were classified as female and those with lower scores as male.

4.4.3 Seasonal and sex-related variation in body mass

For both sexes combined, males only, and females only, the first principal component (PC1; body size index) from a PCA on the three structural measures explained 51.7 %, 51.0%, and 40.6% of the variance, respectively. Mass of adult Tufted Puffins was significantly affected by sex ($F_{1,143} = 45.4$, $P < 0.0001$), body size index (PC1; $F_{1,143} = 21.4$, $P < 0.0001$) and reproductive stage ($F_{2,143} = 11.4$, $P < 0.0001$). The effect of year was not significant ($F_{1,143} = 2.2$, $P = 0.14$), but there was a significant year*reproductive stage interaction ($F_{2,143} = 3.8$, $P = 0.025$). The effects of all other two-way interactions were not significant ($P > 0.3$). The parameter estimate for the effect of sex on body mass was -53.37 (95% C.I.: -69.03, -37.71) indicating that, for a given structural body size, females averaged 53 g lighter than males.

Mass of males was affected by reproductive stage ($F_{2,52} = 6.4$, $P = 0.003$) and body size index ($F_{1,52} = 10.7$, $P = 0.002$). There was no significant year effect ($F_{1,52} = 1.9$ $P = 0.17$), but the interaction between year and reproductive stage was significant ($F_{2,52} = 4.2$, $P = 0.02$). Simple effects tests revealed that the mass of chick-rearing males was lower in 2004 than 2005 (Fig. 4.2a). Mass of female puffins was significantly affected by reproductive stage ($F_{2,88} = 5.2$, $P = 0.008$) and body size index ($F_{1,88} = 12.1$, $P = 0.0008$). Effects of year and all two-way interactions on body mass of females were not significant ($P > 0.3$ for all). Post-hoc analyses revealed that female mass was significantly lower during chick-rearing than late-incubation, whereas mass of pre-lay females did not differ from other reproductive stages (Fig. 4.2b).

4.4.4 Body condition indices

The regression of mass on body size index (PC1) was significant for the pooled-sexes dataset ($F_{1,149} = 63.5$, $P < 0.0001$, $r^2 = 0.3$), the males-only data set ($F_{1,57} = 6.5$, $P = 0.01$, $r^2 = 0.1$) and the females-only dataset ($F_{1,90} = 11.8$, $P = 0.0009$, $r^2 = 0.11$, Fig. 4.3). There was a significant negative linear relationship between residuals output from the pooled-sexes regression and body size index for males ($F_{1,67} = 4.7$, $P = 0.014$) and a negative relationship that approached significance for females ($F_{1,90}$=3.6, $P = 0.061$; Fig. 4.4). These negative relationships indicate a bias in body condition index (residuals) is produced when sexes are pooled for analysis. For the more complex ANCOVA within males, residuals from pooled-sexes were significantly affected by body size index ($F_{1,52} = 5.8$, $P = 0.011$) and by reproductive stage ($F_{2,52} = 6.3$, $P = 0.004$). The effect of year was not significant for males ($F_{1,52} = 1.7$, $P = 0.2$), but the year*reproductive stage interaction was ($F_{2,52} = 4.1$, $P = 0.022$). For females, the effect of body size index on sexes-pooled residuals approached significance ($F_{1,85} = 3.7$, $P = 0.058$) and the effect of reproductive stage was significant ($F_{2,85} = 5.0$, $P = 0.009$). For both sexes, the parameter estimate for the effect of body size on sexes-pooled residuals was negative (means [95% C.I.]; males: $\theta = -10.65$ [-19.5, -1.78]; females: $\theta = -8.94$ [-18.33, 0.33]), indicating a bias towards better body condition in structurally smaller birds of each sex. This bias is an artifact of using sexes-pooled residuals.

4.5 Discussion

4.5.1 Sexual dimorphism and body condition indices

Morphometric measurements of male and female Tufted Puffins in our study were similar to those reported by Piatt and Kitaysky (2002) in the Gulf of Alaska. Although males were larger than females for all three structural features, determining the sex of Tufted Puffins based on morphometrics was only moderately accurate (74%). Because Piatt and Kitaysky (2002) reported significant regional variation in the size of both sexes, our discriminant function may not be applicable outside of our study area. Therefore, we strongly advocate use of genetic sexing techniques, whenever possible.

Our results demonstrate that body condition indices must be calculated separately for male and female Tufted Puffins because pooling the sexes produced significant intra- and inter-sexual bias. This is not surprising given that Shiomi and Ogi (1991) determined male Tufted Puffins are larger and shaped differently than females, with smaller gravimetric proportion of wing muscles to leg muscles. The body condition index we calculated using data for both sexes was biased with structurally smaller birds having inflated indices of body condition (Fig. 4.4). This issue is particularly concerning because it may lead to spurious correlations between body condition index and other factors that vary with body size. Although some investigators separate the sexes prior to analysis (Erikstad et al. 1997) or test for sex-specific relationships between body mass and structural size prior to pooling data (Moe et al. 2002), others assume that scaling mass to structural size accounts for sex-specific differences (Chastel et al. 1995, Tveraa and Christensen 2002). Our results indicate that when using an index of body condition, it is imperative to analyze sexes separately or to test for sex-specific differences prior to pooling data. If structural size is to be used in ANCOVA (Garcia-Berthou 2001), then sex should be included as a factor to avoid bias.

It is often of interest to correlate energy reserves with physiological parameters such as stress hormones (Kitaysky et al. 1999, Lormée et al. 2003) and plasma metabolites (Alonso-Alvarez et al. 2002). This is generally done by scaling body mass to structural size to generate a body condition index. Although indices of body condition based on ratios and residuals are easily calculated and may provide a useful non-destructive tool for estimating energy reserves, studies have revealed that ratio-based indices may produce spurious effects and should be avoided (Jakob et al. 1996, Garcia-Berthou 2001, Green 2001) and that residual based indices should be used with caution (Green 2001).

That said, residuals from ordinary least squares regression of mass vs. structural size may provide an accurate measure of body condition (Golet and Irons 1999, Schulte-Hostedde et al. 2005). Indeed, residuals produced from a regression of body mass on the first principal component extracted from a principal component analysis on a suite of

structural features have been employed by many investigators (Rising and Somers 1989, Chastel et al. 1995, Erikstad et al. 1997, Moe et al. 2002, Tveraa and Christensen 2002). Despite their widespread use, validation of body condition indices in seabird studies is rare (Golet and Irons 1999) and we encourage future investigators to evaluate the efficacy of their methods.

4.5.2 Seasonal mass loss

Growth rates of nestlings in our study are among the highest recorded for this species (reviewed by Piatt and Kitaysky 2002) and were significantly lower in 2004 than 2005. Fledging success was also high in both years. The high rates of nestling growth and fledging success suggest that foraging conditions were generally good for Tufted Puffins during the chick-rearing period. However, the lower fledging mass of nestlings in 2004 suggests that prey availability differed between years. In fact, nestlings diets were of lower quality (lower lipid content) in 2004 than 2005 (CTW, unpubl. data), suggesting that high quality prey was not as available in 2004. The mass of chick-rearing males and females, corrected for body size, was also lower in 2004; however, the difference was only significant for males. In addition, adult males lost a greater percentage of their body mass between late incubation and late chick-rearing than females in both years (Fig. 4.2). Thus, our results are consistent with sex-specific body mass regulation.

Although sex-specific roles in parental care are common among seabirds (Creelman and Storey 1991, Fraser et al. 2002, Paredes et al. 2006), few investigators have determined whether there are commensurate sex-specific differences in loss of body mass. Velando and Alonso-Alvarez (2003) found that female Blue-footed Boobies (*Sula nebouxii*), a species with reverse sexual dimorphism, adjusted body condition in response to experimentally increased nestling demands for food whereas males did not. Conversely, Lormée et al. (2003) found that only male Red-footed Boobies (*Sula sula*), another species with reverse sexual dimorphism, showed a decline in body condition during chick-rearing. Although we did not detect a significant difference between years in seasonal mass loss in female puffins, rates of nestling growth and fledging success

were high in both years and additional investigation is needed to determine if adult females simultaneously adjust their own body condition with rates of nestling provisioning when foraging conditions deteriorate further. Future studies examining provisioning behavior in Tufted Puffin pairs of known-sex would be useful to establish whether sex-specific differences in mass loss are due to greater provisioning effort by males in late chick-rearing. Studies measuring body condition directly via proximate composition analysis would be helpful to determine if males and females differ in their capacity to store lipid reserves, as well as to validate the body condition index used in our study.

In addition to sex-specific differences in adjustment of body mass, species-specific differences have also been reported. Adult Leach's Storm Petrels (*Oceanodroma leucorhoa*) increased provisioning effort when mates were removed, but not enough to compensate for loss of their mate and they did not compromise their own body condition to do so (Takahashi et al. 1999). In contrast, adult Thick-billed Murres (*Uria lomvia*) 'balance' their own body condition with that of their nestlings (Gaston and Hipfner 2006). Differences between species may be attributed, in part, to life-history strategy (Takahashi et al. 1999). Leach's Storm Petrel chicks accumulate large lipid reserves during the nestling period (~60 d) that may provide insurance against stochastic provisioning (Ricklefs and Schew 1994). Murres, however, have a short nestling period (<24 days) and selection apparently favors a strategy of synchronous fledging within subcolonies (Benowitz-Fredericks and Kitaysky 2005). When food is scarce, selection may favor adult murres that deplete endogenous energy reserves during the relatively short nestling stage vs. those that prolong the nestling period and negatively affect chick survival. Tufted Puffin nestlings have highly variable rates of growth in the wild (Piatt and Kitaysky 2002) and are physiologically adapted to modulate metabolic rates in response to nutritional limitation (Kitaysky 1999). Nevertheless, our results suggest that adults, or at least adult males, sacrifice their own body condition in years of diminished nestling growth. Furthermore, Weimerskirch et al. (2001) found that adult Yellow-nosed Albatrosses (*Diomedea chlororhynchos*), another species where chicks accumulate large

lipid reserves, adjust both body condition and chick provisioning rates during years with anomalously high water temperatures. Thus, the amount of endogenous energy reserves nestlings are capable of storing may not be a useful predictor of whether parents adjust body condition when food availability decreases.

Loss of body mass during reproduction can be viewed as the outcome of adaptive compromises between different selective factors (Moreno 1989, Witter and Cuthill 1993). Multi-year studies of seasonal mass loss (Weimerskirch et al. 2001, Gaston and Hipfner 2006, this study), together with experimental manipulations of nestling demands (Erikstad et al. 1997, Takahashi et al. 1999), have revealed sex- and species-specific regulation of energy stores. However, the ultimate cause of interspecific and intersexual variation in regulation remains unclear. Thus, prior to using adult body condition as an indicator of prey availability in any species, it is critical to determine if costs of low food availability are borne by the parent, the offspring, or both. Our results suggest that under conditions of relatively high reproductive success and chick growth rates, adult male Tufted Puffins simultaneously adjust body condition and chick growth rates, whereas females do not. Additional insight into sex-specific regulation of seasonal mass loss in Tufted Puffins may be gained from future studies over a broader range of foraging conditions.

4.6 Acknowledgments

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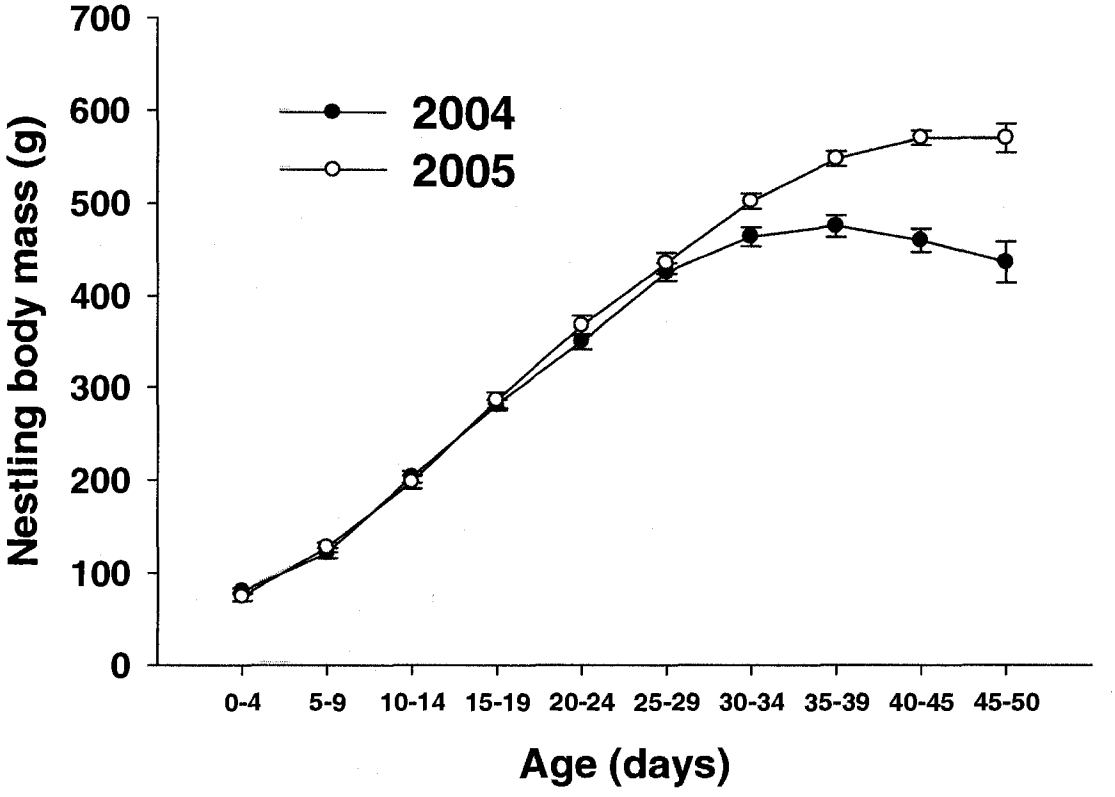

Fig. 4.1 Mean (± SE) mass of nestlings in 5-day age-bins in 2004 ($N = 34$) and 2005 ($N = 43$). Sample sizes in each age-bin averaged 27 (range 13-34) in 2004 and 34 (12-43) in 2005.

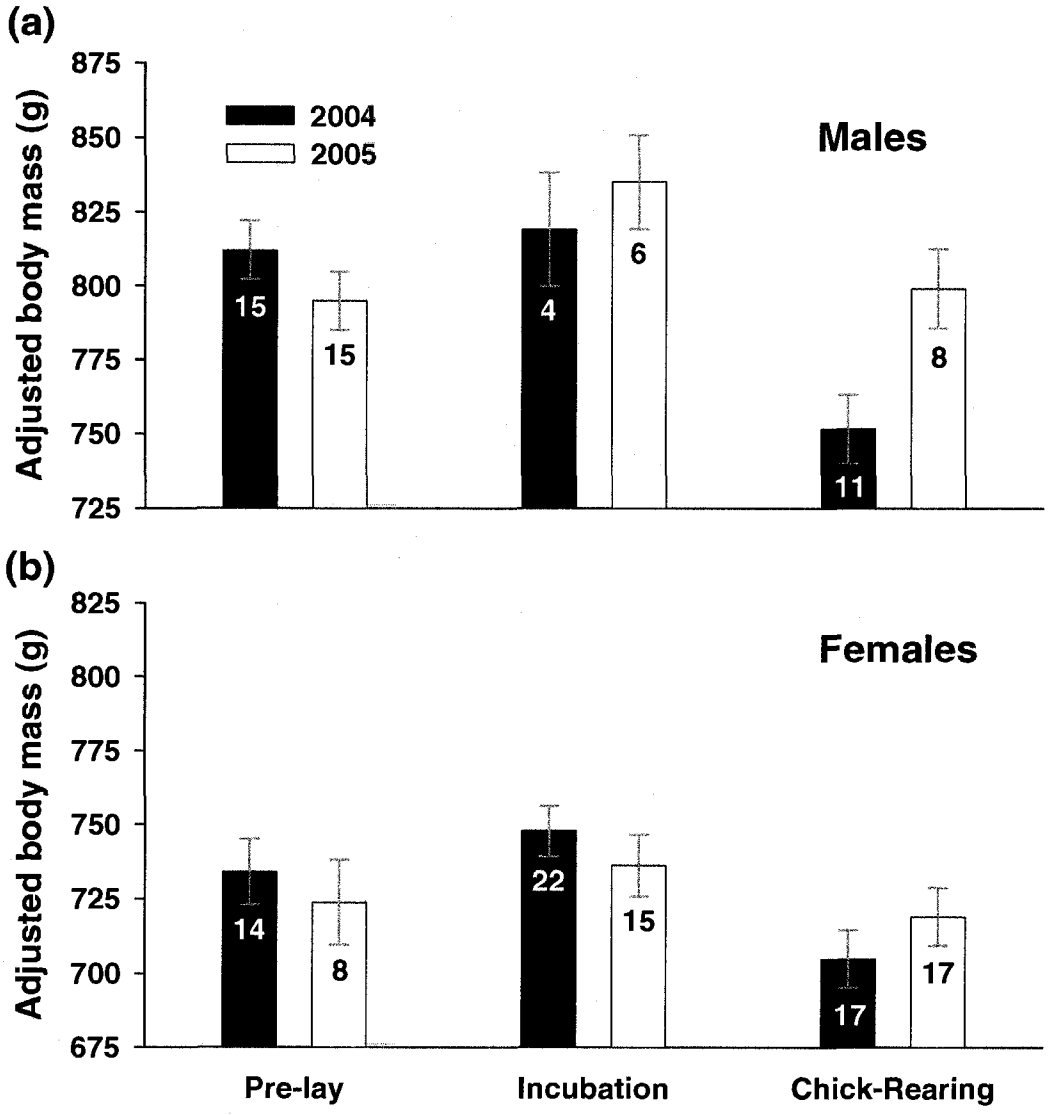

Fig. 4.2 Mean body mass (± SE) adjusted for body size of adult **a** male and **b** female Tufted Puffins during different stages of reproduction in 2004 and 2005. Sample size is shown on each bar.

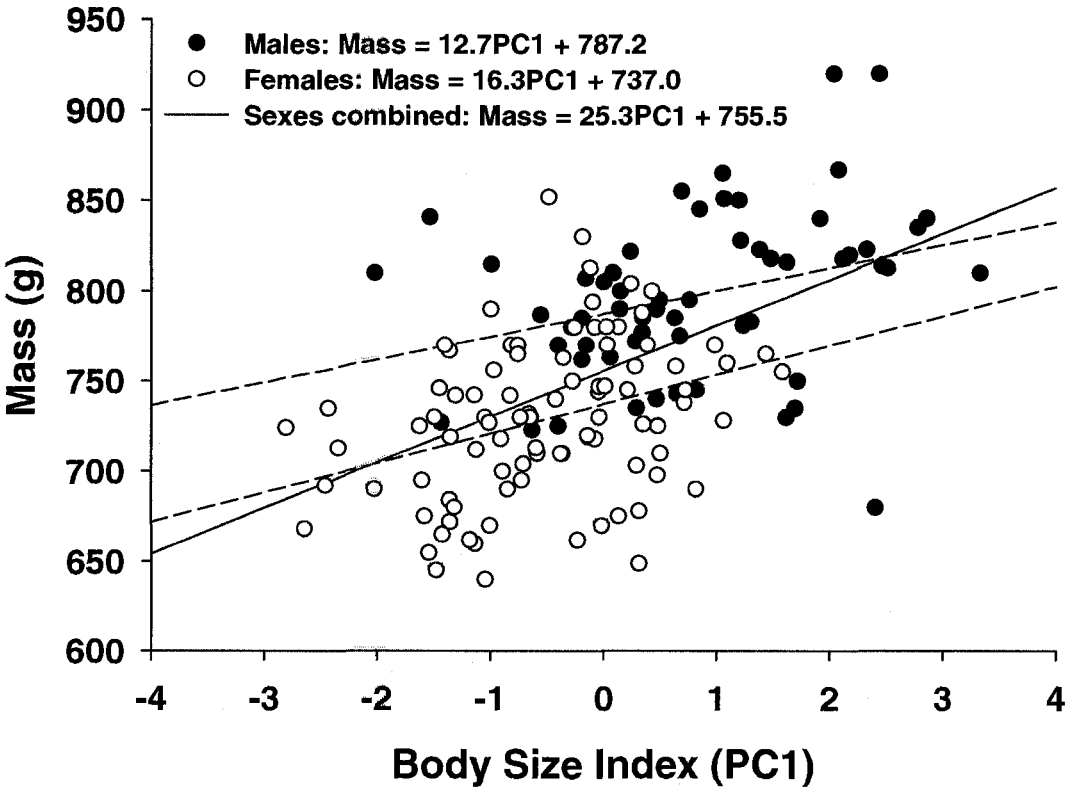

Fig. 4.3 The relationship between adult body mass and body size index in male ($r^2 = 0.1$) and female ($r^2 = 0.11$) Tufted Puffins. The solid line depicts the relationship when both sexes are combined ($r^2 = 0.3$).

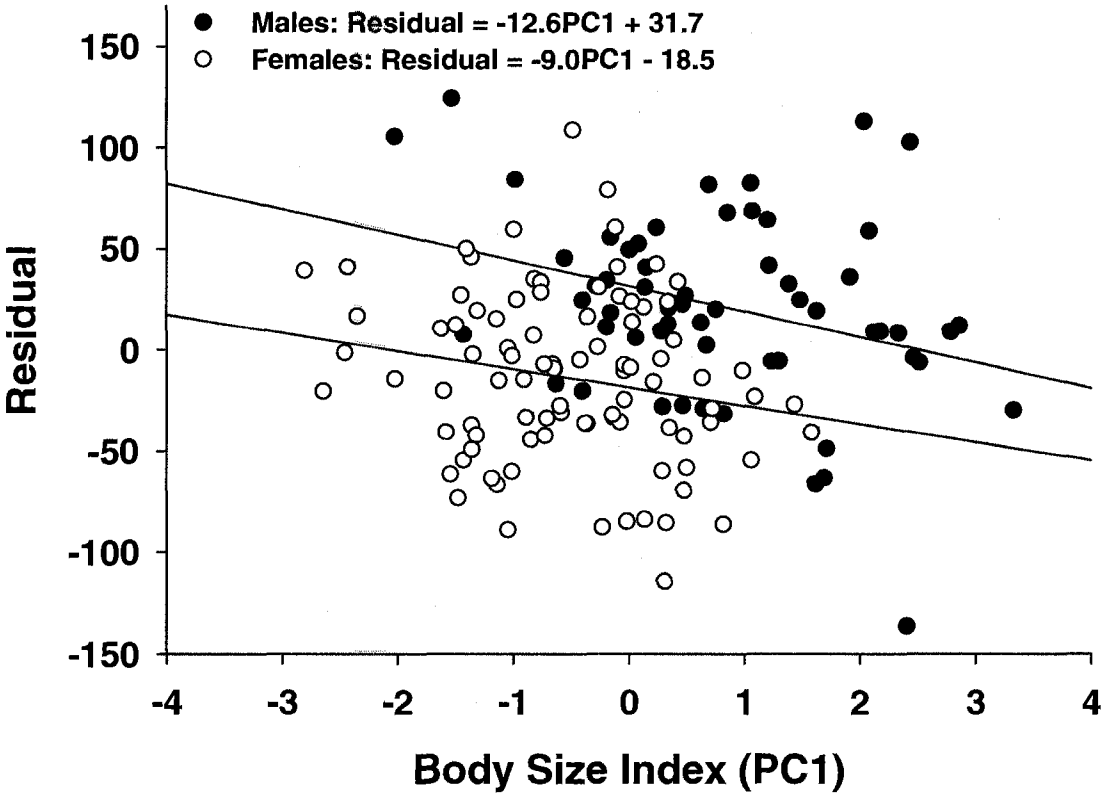

Fig. 4.4 The relationship between body condition index (residuals) and body size index in adult male and female Tufted Puffins. The residuals were output from a regression between adult body mass and body size index using a sexes-combined dataset. A slope of zero would indicate no relationship between body condition index and body size index and thus no intra-sexual bias. The negative slopes indicate pooling sexes produces intra-sexual bias in body condition indices with structurally smaller birds having inflated indices of body condition relative to larger birds within each sex.

Chapter 5: Corticosterone levels of tufted puffins vary with breeding stage, body condition index, and reproductive performance[1]

5.1 Abstract

Corticosterone (CORT) has been proposed as a tool for monitoring the effects of climate-induced changes in prey availability on seabird productivity. Although most studies measure total CORT concentrations, levels of corticosteroid binding globulin (CBG) may also be modulated, thus altering the concentration of CORT available for diffusion into tissues (free CORT). We investigated the seasonal dynamics of CBG, total CORT, and free CORT in breeding tufted puffins (*Fratercula cirrhata*) during two years characterized by high rates of nestling growth and survival. We then compared concentrations of total CORT in this population to levels in chick-rearing puffins at another colony during two years with low productivity. At the high productivity colony, levels of CBG, total baseline CORT, free baseline CORT, and total maximum CORT were all higher prior to egg-laying than during late incubation and late chick-rearing. Levels of CBG were positively correlated with body condition index (BCI) and free baseline CORT was negatively correlated with BCI. Total baseline levels of CORT during chick-rearing were two to four times higher at the colony with low rates of nestling growth and survival. Our results suggest the seasonal trend in baseline CORT levels is either driven by, or can be overridden by, changes in prey availability. Given the negative effects associated with chronic elevation of CORT, our results indicate the cost of reproduction may be higher during years characterized by low productivity.

[1]Previous version submitted as: Williams CT, Kitaysky AS, Kettle AB, Buck CL (2007, submitted) Corticosterone levels of tufted puffins vary with breeding stage, body condition index, and reproductive performance. General and Comparative Endocrinology.

5.2 Introduction

Primary production and ecosystem dynamics in the North Pacific are affected by multi-year invasion of nutrient-poor warm surface waters associated with the El Nino Southern Oscillation (ENSO; Zamon and Welch, 2005) and by periodic shifts between warm and cold regimes that occur on a multi-decadal time scale (the Pacific Decadal Oscillation or PDO; McGowan et al., 1998). These changes in ocean climate alter the abundance and distribution of forage fishes, and consequently affect apex predator populations (Anderson and Piatt, 1999). Seabirds are often proposed as useful indicators because their productivity is tightly linked to prey availability (Cairns, 1987; Suryan et al., 2002; Frederiksen et al., 2006). However, productivity of seabirds also depends on local oceanography and weather conditions (e.g. Springer, 2006) necessitating sampling a large number of colonies over an enormous geographic area to disentangle basin-wide effects of climate shifts from changes in local oceanography. Traditional seabird monitoring programs that require observers at colonies throughout the breeding season are often prohibitively expensive over such a large spatial scale. Field endocrinology may provide an economically feasible tool for monitoring the effects of changing ocean climate because blood samples can be obtained on short visits to colonies.

In birds, activity of the hypothalamus-pituitary-adrenal (HPA) axis reflects predictable life history events and unpredictable environmental conditions (Wingfield et al., 1997; Romero, 2002). Activation of the HPA axis results in increased plasma concentrations of corticosterone (CORT), a steroid released by the adrenal cortex, which stimulates mobilization of stored energy reserves and elicits behavioral changes that promote survival (reviewed in Sapolsky et al., 2000). Because birds often respond to environmental stressors by increasing circulating levels of CORT, it has been promoted as a useful monitoring tool (Wikelski and Cooke, 2006). Furthermore, applying a standardized capture and handling protocol permits researchers to measure the capacity of an individual to respond to acute stress, providing information on past exposure to stressors (Kitaysky et al., 2007). Most field studies measure total levels of circulating CORT in the blood which includes hormone bound to corticosteroid binding globulin

(CBG) and unbound or free CORT. However, it has been argued that only free hormone is available to tissues and may therefore be a more meaningful measure of physiological stress (Breuner and Orchinik, 2002; Love et al., 2004).

Total baseline levels of CORT have been shown to correlate with reproductive success and body condition in black-legged kittiwakes (*Rissa tridactyla*; Kitaysky et al., 1999; Buck et al., 2007) as well as with food availability in common murres (*Uria aalga*; Kitaysky et al. 2007). Kitaysky et al. (2007) also found that total maximum stress-induced levels of CORT in murres were correlated with food availability in the recent past (one month prior). These studies provide support for the concept of using CORT as a monitoring tool in seabirds, although Lanctot et al. (2003) suggest that CORT is a good indicator of forage availability in only some situations.

Activation of the HPA axis is not without costs, however, because chronic elevation of glucocorticoids can have deleterious effects including muscle wasting, impaired cognitive abilities, and compromised immune function (Saino et al., 2003; Sapolsky et al., 2000). Seabirds are relatively long-lived and have low annual fecundity; it is possible that in some species adults are unwilling to endure greater reproductive costs when food availability is low. Under this scenario, consequences of reduced prey availability are borne entirely by their offspring and adults may even elect to forego reproduction entirely rather than experience greater reproductive costs. This appears to be the case for some adult seabirds that seem unwilling to sacrifice body condition to maintain chick provisioning rates when flight costs are experimentally increased (e.g. Saether et al., 1993; Weimerskirch et al., 1999; Duriez et al., 2000). Adults may also avoid the costs associated with chronic elevation of CORT by prioritizing their own maintenance over the survival of their young. If this is the case, then adult CORT levels may not accurately reflect productivity.

The adrenocortical response to environmental perturbations is likely species-specific and the role of CBG in modulating free CORT has only recently been addressed in seabirds (Dempsey, 2006; Shultz and Kitaysky, 2008). Furthermore, plasma CORT and CBG concentrations may be affected by stage of reproduction which could confound

use of CORT as a measure of physiological stress (Breuner and Orchinik, 2002; Romero, 2002; Love et al., 2004). In this paper, we investigate total CORT, CBG, and free CORT in free-living tufted puffins (*Fratercula cirrhata*) during three stages of reproduction: prior to egg laying, late incubation, and late chick-rearing. We also include body condition index as a covariate in our analyses to determine whether CORT and CBG vary with endogenous stores of energy. We then compare total baseline and maximum stress-induced CORT levels in chick-rearing adults from a high productivity colony to levels in adults captured at a different colony during years of low productivity. We predicted that total CORT would negatively correlate with nestling growth and survival rates, indicating costs associated with low food availability are incurred by adults as well as nestlings.

5.3 Methods

5.3.1 Study site and species

This study was carried out on East Amatuli Island (EAI) in the Barren Islands group, AK (58°55'N, 152°00'W) in 1996-1997 and on Chiniak Island (CI) in Chiniak Bay on the northeast side of Kodiak Island, AK (57°40'N, 152°20W) in 2004-2005. At both locations, tufted puffins nest colonially in single-pair burrows excavated in the soil. Puffins are monogamous and exhibit bi-parental care with males and females sharing incubation and chick-rearing duties. A single egg is laid between late May and early June, hatching occurs from mid to late July, and chicks fledge between late August and early September. Rates of nestling growth and age at fledge varies widely across years and colonies (Piatt and Kitaysky, 2002), presumably due to differences in prey availability.

5.3.2 Colony productivity and growth rates

We began collecting data on nestling growth rates and fledging success on 23 July in 1996 ($N = 73$ nestlings monitored), 28 July in 1997 ($N = 57$), 24 July in 2004 ($N = 34$), and 22 July in 2005 ($N = 44$). Nestlings that could not be reached through the entrance were accessed using holes excavated in previous years and sealed with either plywood or flat rocks. We weighed chicks using spring scales (± 2 g) and measured flattened wing-

length from the wrist to the wingtip (± 1mm) every 4-5 days throughout the nestling period. The final day we examined burrows for the presence of a chick and weighed chicks still remaining in the nest was between 10-12 September in all years. When hatch date was unknown, we estimated age using a wing-length vs. age regression derived from known-age nestlings on CI. We calculated growth rate for each nestling as the slope of the linear regression equation relating mass and age between ages 10 and 30 days, the near-linear portion of the growth curve (Gjerdrum et al. 2003). Data from known-age nestlings were lacking for EAI; therefore, we estimated age based on known-age nestlings from CI. Because of the lower growth rates on EAI (see results), nestling age may have been underestimated for nestlings on this island. However, expanding or contracting the age-range used in our analysis had little effect on estimates of growth rates for EAI chicks, indicating our analysis was robust against slight bias in age estimates. We defined fledging success as the number of chicks reaching a minimum wing-length of 130 mm per egg hatched (Gjerdrum et al., 2003) and assumed that chicks died if they disappeared before attaining this minimum.

5.3.3 Capture and blood sampling

To minimize the effects of diel variation in CORT and/or CBG levels, all birds were captured between 10:00 and 17:00. At EAI, we captured adults by hand in their burrows during early chick-rearing (1996: 28 July; 1997: 1 August) and determined breeding status at the time of captures; only actively breeding birds were sampled. At CI, we captured adults during four time periods: prior to egg-laying (22 May-2 June), late incubation (1-11 July), early-chick rearing (2005 only; 4-12 August), and late chick-rearing (23 August-4 September). During egg-laying and incubation, puffins were captured with a 7x10m net draped over a cluster of 20-30 burrow entrances. We were unable to confirm breeding status of birds captured using this method. However, all birds captured during incubation had a brood patch. During early and late chick-rearing, adults were captured by hand in their burrows. We captured most adults in late chick-rearing immediately after they delivered bill-loads of food to their young. If an adult was

captured more than once, we included only data from the first capture in our analysis to ensure all observations were independent.

We obtained blood samples by puncturing either the alar or tarsal vein with a 23-guage needle and collected blood in heparinized capillary tubes. An initial blood sample was taken within 3 min of reaching into burrows or capture in a net, before circulating levels of CORT increase in response to handling induced stress (Romero and Reed, 2005). We did not find a significant relationship between time after capture and CORT concentrations within 0-3 min; therefore, we assume these samples are indicative of baseline levels. Birds were restrained in cloth bags and additional samples were collected at 10, 30, and 50 min. We defined the maximum stress-induced CORT level as the highest concentration measured across the time-series of samples (following Kitaysky et al., 2007). Only baseline samples were obtained from birds captured during early chick-rearing on CI. Blood was transferred immediately into 0.5 or 1.5 ml microcentrifuge tubes which were stored on ice for several hours until they were centrifuged and separated. Plasma and blood cells were stored frozen (-70°C) until laboratory analyses were performed. We extracted DNA from blood cells using a DNeasy tissue kit (QIAGEN Inc., Valentia, CA) and determined sex according to the methods of Griffiths et al. (1998). We did not determine sexes of puffins captured on EAI.

At CI, we measured adult wing-chord length using a ruler (± 1mm), and bill and straight tarsus lengths using dial calipers (± 0.1mm). Body mass was determined using a spring scale (± 2g). We regressed mass against the first principal component (PC1) of a principal component analysis on the three morphometric measures and used the output residuals as a body condition index (BCI; see details in Williams et al., 2007). We calculated BCI separately for each sex because the relationship between mass and structural size is sex-specific in this species. The relationship between mass and PC1 was determined for each sex using datasets consisting of 69 males and 107 females (Williams et al., 2007). Puffins captured at EAI were not sexed and BCI was not calculated.

5.3.4 Laboratory techniques

For each plasma sample, we determined CORT concentration in duplicate following extraction in dichloromethane using radioimmunoassay as detailed in Wingfield et al. (1992). We determined recovery values and used these to adjust final assayed concentrations of CORT. Mean recovery was 92 ± 7 % (SD). Four assays were conducted; inter-assay variation was 7% and intra-assay variation ranged between 2-3%. For puffins captured on CI in 2005 and 2006, we randomly selected between 8 and 12 plasma samples from each stage of reproduction (excluding early chick-rearing) in each year and determined CBG concentrations as described in Love et al. (2004). We determined optimal incubation time (2 h), final plasma concentration (1:450) and CORT affinity (Kd = 2.21 ± 0.257). Exogenous CORT was stripped from plasma by incubating each 10 µl sample with 20 µl dextran-coated charcoal solution for 20 min at room temperature prior to centrifugation at 4000 rpm for 10 min at 4°C. We analyzed samples for total binding in triplicate and simultaneously determined non-specific binding in duplicate using unlabeled CORT. Following incubation, we separated bound and free radioligand using rapid vacuum filtration (Brandel Harvester) over glass fiber filters (Whatman GF/B, soaked in 25mM Tris with 0.3% PEI for 1 h before filtering). All plasma samples used for determination of CBG concentrations were obtained from blood samples collected within 3 min of capture. Free CORT was estimated using the equation of Barsano and Baumann (1989). All samples were run in a single assay; intra-assay variation was 6%.

5.3.5 Statistical analyses

We performed all statistical analyses using SAS 9.1 (SAS Institute 2006). We tested for differences in nestling mortality between all four years (all pairwise comparisons) using continuity-adjusted chi-squared tests with alpha adjusted to 0.0083 using a Bonferonni correction. We compared rates of nestling growth between all four years using ANOVA followed by post-hoc Tukey HSD tests. Because of the large number of chicks still remaining in their nests on the day of final nest checks in 1996 and

1997 at EAI (see results), we compare fledging masses between years only at CI (using a Students t-test).

For puffins captured on CI, we investigated the seasonal dynamics of total baseline CORT, free baseline CORT, CBG capacity, and total maximum CORT using four separate ANCOVAs. We log-transformed total baseline CORT, total maximum CORT, free baseline CORT, and CBG capacity to meet the assumptions of normality and homoscedasticity required for parametric tests. Two log-transformed values obtained for free baseline CORT proved to be outliers and were subsequently excluded. Exclusion of these two observations did not affect the results of statistical tests. We included the main effects year, sex, and stage of reproduction along with body condition index as a continuous covariate. The initial global model included all two-way interactions; interaction terms were subsequently dropped when $p > 0.10$. Similar to previous seabird studies (Buck et al., 2007; Kitaysky et al., 2007), we found no evidence for an effect of sex on free CORT, total CORT, or CBG, and therefore we subsequently excluded sex from all models. Significant stage of reproduction effects were examined using post-hoc Tukey HSD tests.

We compared total baseline CORT in adults captured during early chick-rearing to adults captured in late chick-rearing at CI in 2005 using a Mann-Whitney U test. We compared chick-rearing levels of total baseline and total maximum CORT between all four years using Kruskal-Wallis tests (because variance was unequal between years), followed by Tukey HSD tests. For this analysis, adults were sampled during late chick-rearing at CI and early chick-rearing at EAI. However, because baseline CORT levels were not significantly different between early vs. late chick-rearing birds at CI in 2005 (see results) we assumed that observed differences were not a function of stage of chick-rearing.

5.4 Results

5.4.1 Colony productivity and growth rates

Fledging was delayed at EAI relative to CI. At EAI, 25 % (18 of 73) and 39 % (22 of 57) of the study chicks remained in the nest on the final day of monitoring in 1996 and 1997, respectively. In contrast, only 3 % (1 of 34) and 5% (2 of 44) of chicks were still in their nests on the final day of monitoring at CI in 2004 and 2005. At EAI, 63 % (46 of 73) and 30 % (17 of 57) of the monitored nestlings were either found dead or were presumed to have died (disappeared from nest when wing chord < 130 mm) in 1996 and 1997, respectively. Mortality was lower on CI in 2004 (12 %; 4 of 34) and in 2005 (5 %; 2 of 44), although the difference between 2004 on CI and 1997 on EAI was not significant ($X^2 = 2.96$, $p = 0.09$). Mortality rates did not differ ($p = 0.45$) between years on CI, but on EAI mortality was significantly higher ($p < 0.0003$) in 1996 than in 1997.

The mean (± SE) rate of mass increase during the linear growth phase was 4.4 ± 0.4 g/day ($N = 59$) in 1996, 8.7 ± 0.6 g/day ($N = 48$) in 1997, 14.3 ± 0.5 g/day ($N = 28$) in 2004, and 16.2 ± 0.5 g/day ($N = 41$) in 2005. Growth rates of puffin nestlings were significantly different between years ($F_{3,174} = 121.18$, $p < 0.0001$). Post-hoc Tukey tests indicated growth was significantly ($p < 0.05$) more rapid in 2004 and 2005 than in 1996 and 1997; growth in 1997 was also significantly more rapid than in 1996. Although growth rates at CI did not differ between 2004 and 2005, nestlings obtained a higher peak mass and had lower levels of mass recession in 2005 which ultimately led to a higher mass at fledging in that year (2004: 467.3 ± 11.9 g, $N = 28$; 2005: 563.6 ± 8.2 g, $N = 38$; $t = -6.87$, $p < 0.0001$; Fig. 5.1).

5.4.2 CBG and total and free CORT

For CI adults, total baseline CORT was significantly affected by stage of reproduction ($F_{2,94} = 22.28$, $p < 0.0001$), but not by BCI ($F_{1,94} = 3.35$, $p = 0.07$), or by year ($F_{1,94} = 0.76$, $p = 0.38$). Total baseline CORT was significantly higher prior to egg laying compared to late incubation and late chick-rearing (Tukey HSD test, $p < 0.05$, Fig. 5.2a). CBG capacity was significantly affected by stage of reproduction ($F_{2,56} = 11.31$, p

< 0.0001) and was positively affected by BCI ($F_{1,56} = 7.51$, $p = 0.008$); the effect of year was not significant ($F_{1,56} = 0.82$, $p = 0.37$). Similar to total baseline CORT, CBG capacity was higher during pre-lay compared to late incubation and late chick-rearing (Tukey HSD test, $p < 0.05$, Fig. 5.2b). Free baseline CORT was also significantly affected by stage of reproduction ($F_{2,54} = 9.74$, $p < 0.001$), and was negatively affected by BCI ($F_{1,54} = 12.13$, $p = 0.001$); there was no significant year effect ($F_{1,54} = 0.04$, $p = 0.83$). Tukey HSD test revealed that free baseline CORT was significantly higher prior to egg-laying than during late incubation or late chick-rearing ($p < 0.05$, Fig 5.2c). Capture and handling protocol resulted in a robust stress response during all time-periods (Fig. 5.3). Total maximum CORT was significantly affected by stage of reproduction ($F_{2,94} = 10.61$, $p < 0.0001$). The effect of year on maximum CORT was also significant ($F_{1,94} = 5.44$, $p = 0.02$) whereas the effect of BCI was not ($F_{1,94} = 0.22$, $p = 0.64$). Post-hoc Tukey tests revealed pre-lay puffins had higher maximum CORT than birds captured in late incubation or late chick-rearing ($p < 0.05$; Fig. 5.2d). Total maximum CORT appeared to be elevated during the chick-rearing period of 2004.

At CI in 2005, total baseline CORT in early chick rearing (2.7 ± 0.7 ng/ml (SE), $N = 6$) was not significantly different from levels measured in late chick-rearing (6.1 ± 1.5 ng/ml, $N = 16$; $U = 51$, $p = 0.2$). Total Baseline CORT during chick-rearing was significantly different between the four years ($F_{3,41} = 10.34$, $p < 0.0001$). Post-hoc Tukey tests revealed that total baseline CORT levels from EAI in 1996 and 1997 were significantly higher than levels from CI in 2004 and 2005 ($p < 0.05$; Fig. 5.4). Total maximum CORT during chick-rearing differed significantly between the 4 years ($F_{3,40} = 6.36$, $p = 0.001$). Tukey tests revealed that total maximum CORT levels in 1997 and 2004 were significantly higher than in 2005 ($p < 0.05$; Fig. 5.4). Levels of total baseline CORT were higher in the two years characterized by low rates of nestling growth, but there was no clear relationship between total maximum CORT and chick growth (Fig. 5.4).

5.5 Discussion

We examined whether total CORT, free CORT, and/or CBG were correlated with stage of reproduction, body condition index, or colony productivity in a relatively long-lived seabird, the tufted puffin. At CI, tufted puffins exhibited baseline and maximum levels of total CORT that were more than 2.5-times higher prior to egg-laying compared to late incubation and late chick-rearing. The observed pattern for CBG mirrored that of total CORT, yet free CORT was also significantly elevated during the pre-lay period. Although plasma CORT levels of adult puffins from CI suggest a stage-specific trend, CORT levels from chick-rearing birds captured at EAI during two years characterized by low rates of nestling growth and survival indicate the seasonal trend in total CORT is driven by, or can be overridden by, changes in prey availability. Elevated total baseline CORT levels in chick-rearing birds captured at EAI during the low-productivity years are consistent with the hypothesis that the cost of reproduction changes with environmental conditions.

5.5.1 Seasonal variation in CORT and CBG: endogenous or exogenous effects?

The seasonal dynamics of CBG and total and free CORT in tufted puffins was similar between the two years at CI, yet it is not clear if this is indicative of an endogenous seasonal rhythm. For other seabird species, the data are sparse and equivocal. In a five-year study of common murres, Kitaysky et al. (2007) found no consistent seasonal patterns for total baseline or total maximum CORT. During a one-year study of red-footed boobies (*Sula sula*), Lormée et al. (2003) found that total baseline CORT levels were lower during pre-lay than during incubation and chick-rearing. No consistent differences were found in total CORT, free CORT, or CBG levels between incubation and chick-rearing stages for either black-legged kittiwakes (Shultz and Kitaysky, 2008) or red-legged kittiwakes (*Rissa brevirostris*; Dempsey 2006). Additionally, total baseline and maximum CORT levels during the chick-rearing period at our low productivity site were comparable to levels observed prior to egg-laying at our high productivity colony. Assuming low productivity was driven by low food availability, as is typical for puffin

species (Barrett, 2002; Durant et al., 2006; Frederiksen et al., 2006), our results indicate that a shortage of food during chick-rearing could mask an endogenous seasonal rhythm in total CORT, if one exists.

It is also possible that low food availability during the pre-lay period in 2004 and 2005 was the proximate driver of elevated levels of total CORT we observed prior to egg-laying. Assuming that limited food availability elicited elevated CORT during pre-lay, the physiological basis for simultaneously increasing plasma CBG levels is not apparent. However, CBG is not solely a glucocorticoid transporter; CBG also functions as a sex-steroid transporter, is thought to be internalized in some cells, and may activate intracellular second-messenger systems (reviewed in Breuner and Orchinik, 2002). CBG is also cleaved by activated neutrophils, releasing CORT at sites of inflammation (Pemberton et al., 1988) and elevated CBG may therefore be useful in providing a greater plasma reservoir of CORT. Given the multiple roles of CBG, long-term studies and captive experiments are needed to disentangle ultimate rhythms in CBG and CORT from effects of proximate factors.

Love et al. (2004) suggested that increased free baseline CORT during the chick-rearing phase in starlings may be adaptive and necessary to increase the foraging activity of parents so they can meet the energetic demands of chicks. In contrast, levels of total and free baseline CORT in chick-rearing puffins from CI are the same as in birds captured during incubation even though BCI declines between late-incubation and late chick-rearing (Williams et al., 2007). Alcids typically lose body mass during reproduction and this mass loss is thought to be primarily due to depletion of lipid stores (e.g. Niizuma et al., 2002). It has been suggested that loss of body mass during chick-rearing may be an intrinsic process in seabirds (Jones, 1994). Nevertheless, studies indicate that adult body mass is positively correlated with parental provisioning rate and with rates of nestling growth and survival in some species (Weimerskirch et al., 2001; Gaston and Hipfner, 2006; Williams et al., 2007). Ultimately, whether a concomitant increase in CORT is driven by the loss of body mass may depend on the degree of mass loss and depletion of endogenous lipid stores. In fasting penguins, for example, energy is

derived primarily from lipid stores and CORT levels remain low and stable until lipids are depleted beyond some threshold level at which point CORT levels and protein catabolism increase (Robin et al., 1998).

Our results indicate mass loss during chick-rearing is not necessarily associated with an increase in total CORT in puffins. We postulate that in low productivity years, food availability is reduced and adults lose more body mass and deplete their lipid reserves, eventually resulting in significantly elevated CORT levels. However, we lack data on adult BCI from EAI so we are unable to determine whether elevated CORT at this location was accompanied by low BCI. Additionally, total and free levels of CORT were elevated during the pre-lay period at CI yet BCI of birds during the pre-lay period was not different from that of incubating adults (Williams et al., 2007). This lack of concordance between CORT and BCI suggests a sliding set-point exists for body mass or body condition that depends on the stage of reproduction.

5.5.2 Free vs. total CORT

The free-hormone hypothesis posits that only unbound CORT is available to tissues (Mendel, 1989) and may therefore be a more valid measure of physiological stress (Breuner and Orchinik, 2002). For instance, Love et al. (2004) found free baseline CORT was higher in European starlings (*Sturnus vulgaris*) that abandoned nests compared to those that did not abandon, yet total baseline and total stress-induced levels showed no such relationship. Although we found no evidence that CBG altered the seasonal dynamics of CORT at CI, we did find a positive relationship between CBG and BCI which resulted in a negative relationship between free CORT and BCI. Assuming our BCI is an accurate measure of stored energy reserves, this result is consistent with studies that found nutritional state affects CBG capacity and consequently free CORT. Rat pups subject to perinatal food restriction (Léonhardt et al., 2002), white-crowned sparrows (*Zonotrichia leucophrys gambelii*) subjected to fasts (Lynn et al., 2003), and red-legged kittiwake chicks subject to a low calorie low-protein diet (Dempsey, 2006) all exhibit reduced CBG levels compared to controls. Ultimately, long-term studies are needed to

determine whether free CORT reflects seabird productivity more accurately than does total CORT.

5.5.3 Elevated CORT and the cost of reproduction

Experimental manipulations of reproductive effort in long-lived seabirds demonstrate costs in terms of adult survival (Golet et al., 1998) and future fecundity (Wernham and Bryant, 1998) but little is known regarding adjustment of costs in response to current environmental conditions. Because chronically elevated CORT has negative physiological consequences, it may provide insight into the cost of reproduction. Elevated CORT in murres is correlated with a greater probability of skipping breeding in subsequent years and a lower probability of survival (Kitaysky et al., 2007). Furthermore, experimental elimination of reproductive effort in another long-lived seabird, the black-legged kittiwake, resulted in reduced plasma CORT concentrations and increased survival (Golet et al., 2004). Levels of total CORT in chick-rearing puffins were much higher during years characterized by low rates of nestling growth and survival. However, we were unable to detect a difference in total baseline CORT between the two low productivity years, despite the relatively large differences in nestling growth and survival rates between these years. Though the difference in CORT was non-significant, the result was opposite to what we expected: baseline CORT was lower in the lowest productivity year (1996) than in the year of moderate productivity and chick growth (1997). These results may indicate a non-linear relationship between CORT and productivity, which could limit the utility of CORT as a monitoring tool. Alternatively, this non-significant trend may be a function of our small sample size for CORT measures in both years at EAI.

Assuming that chronic elevation of CORT negatively affects survival and/or future fecundity, results of our study and previous studies (e.g. Buck et al., 2007; Kitaysky et al., 2007) indicate the cost of reproduction in murres, kittiwakes, and puffins may change with current foraging conditions. In contrast, some species may exhibit a fixed cost of reproduction. Handicapping adult prions and petrels with leg weights results

in increased foraging trip duration and decreased chick meal mass, but adult body mass was unaltered (Saether et al., 1993; Weimerskirch et al., 1999; Duriez et al., 2000). However, in the yellow-nosed albatross (Weimerskirch et al., 2001) and the thick-billed murre (*Uria lomvia*; Gaston and Hipfner, 2006) adult body mass is postively correlated with productivity supporting the premise that the cost of reproduction may be higher in low productivity years. We contend that elevated CORT in low productivity years also provides evidence that reproductive costs reflect changing environmental conditions. However, more studies are needed to determine whether CORT and/or body mass (or BCI) are actually indicative of a cost to future reproductive output.

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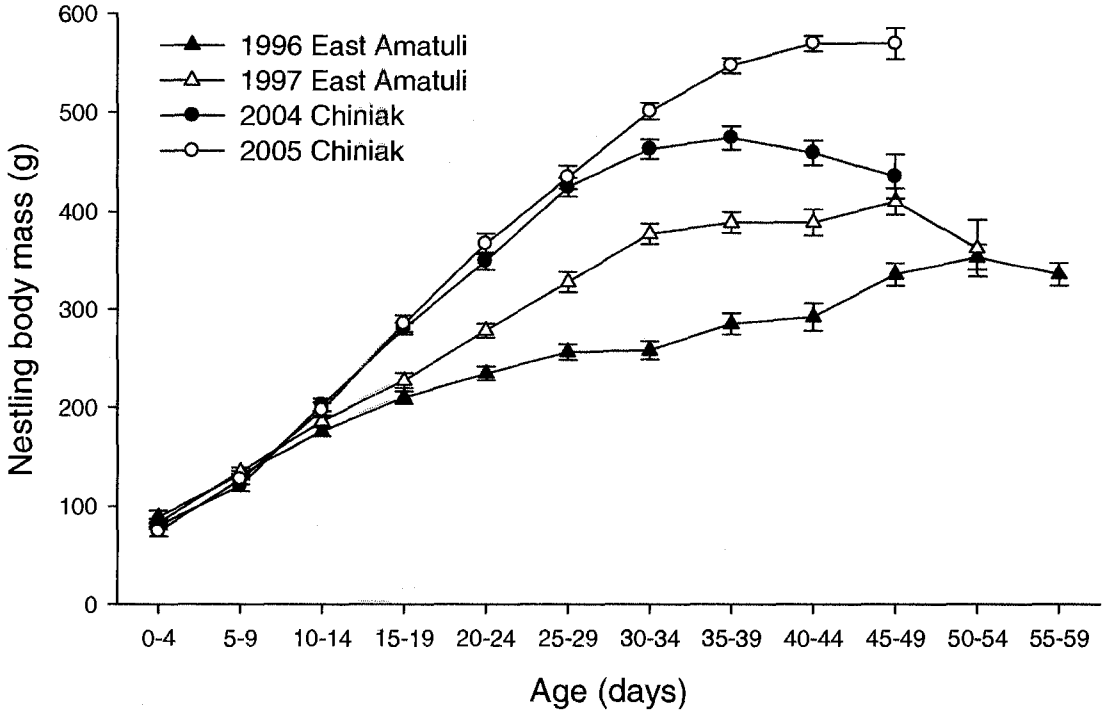

Fig. 5.1 Mean mass (± SE) of nestlings in 5-day age-bins at East Amatuli Island in 1996 (n = 73) and 1997 (n = 58), and at Chiniak Island in 2004 (n = 34) and 2005 (n = 43). Sample sizes in each age-bin averaged 34 (range 10-55) in 1996, 33 (9-47) in 1997, 27 (13-34) in 2004 and 34 (12-43) in 2005.

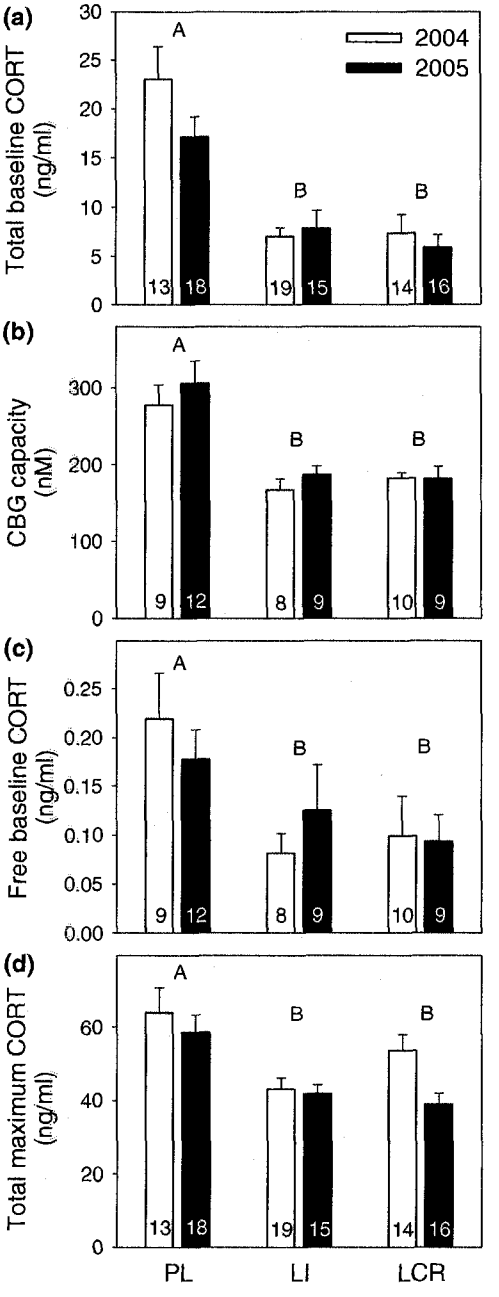

Fig. 5.2 Plasma levels (± SE) of (a) total baseline CORT, (b) CBG, (c) free baseline CORT, and (d) total maximum CORT in tufted puffins captured at Chiniak Island in 2004 and 2005 during three breeding stages: prior to egg laying (PL), late incubation (LI), and late chick-rearing (LCR). Sample sizes for each year and breeding stage are denoted on the bars. Different letters above the bars designate significant differences ($p < 0.05$) between breeding stages.

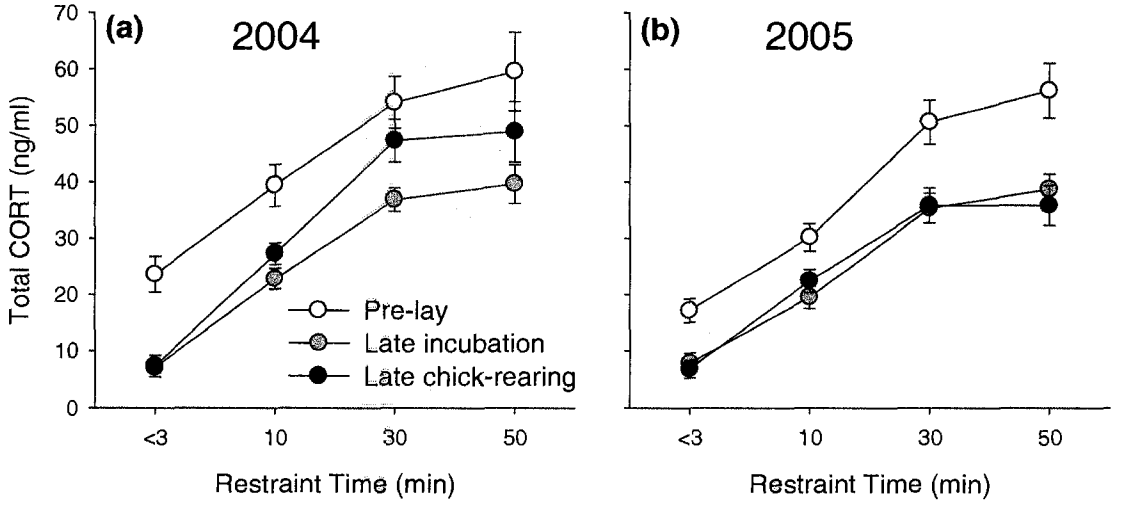

Fig. 5.3 Seasonal pattern in stress-induced levels of CORT (mean ± SE) of adult tufted puffins captured in (a) 2004 and (b) 2005 during three breeding stages: pre-lay, late incubation, and late chick-rearing.

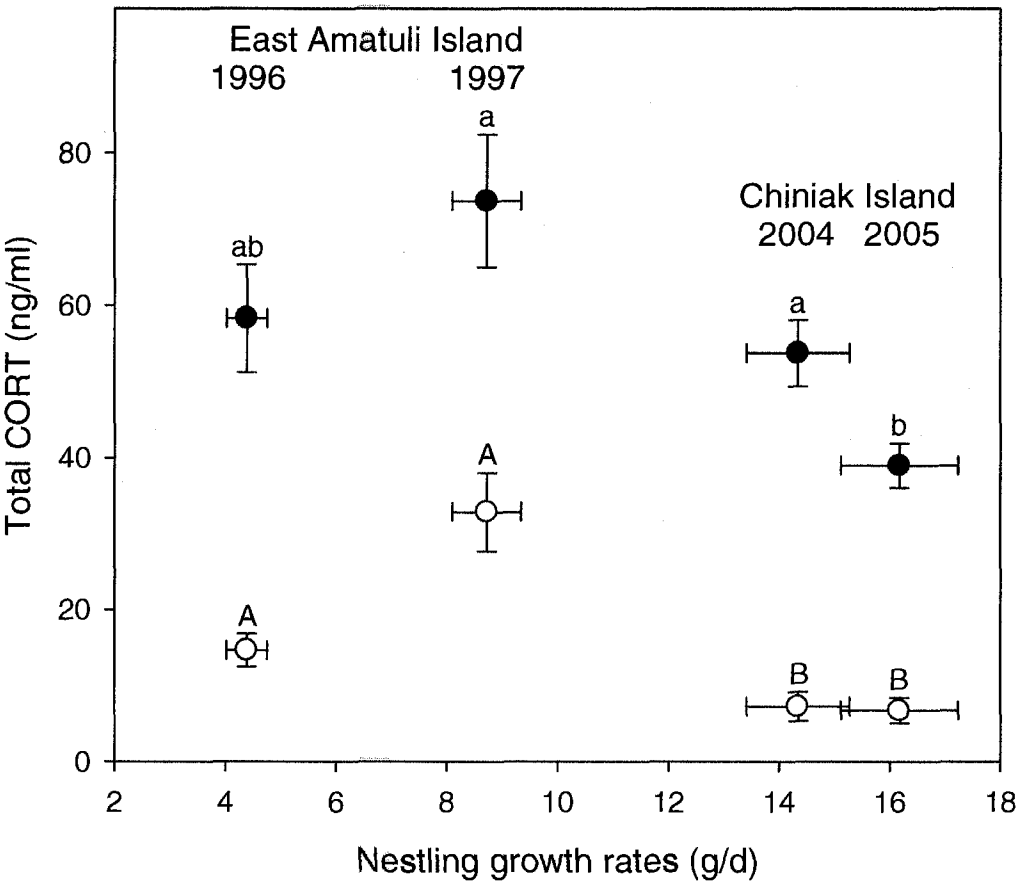

Fig. 5.4 Mean (± SE) total plasma baseline (open circles) and maximum (filled circles) CORT in chick-rearing tufted puffins vs. mean (± SE) rates of nestling growth. Data are from East Amatuli Island in 1996 (baseline and maximum CORT: $N = 6$) and 1997 (baseline CORT: $N = 6$; maximum CORT: $N = 5$) and from Chiniak Island in 2004 ($N = 14$) and 2005 ($N = 16$). Different upper-case letters above circles indicate significant differences ($p < 0.05$) in mean total baseline CORT whereas different lower-case letters designate significant differences in mean total maximum CORT.

Chapter 6. Food restricted tufted puffin (*Fratercula cirrhata*) nestlings increase begging behavior without modulating total or free corticosterone[1]

6.1 Abstract

In some bird species, corticosterone (CORT) appears to play a role in the control of begging behavior of chicks. Because of the potentially high costs associated with chronic elevation of CORT, it has also been proposed as a mechanism to ensure begging is an honest signal. We determined the effects of moderate food restriction (50% of high calorie treatment) on begging behavior and baseline levels of both total and 'free' unbound CORT in tufted puffin (*Fratercula cirrhata*) nestlings. We also measured total and free CORT in free-living tufted puffin chicks to determine if hormone levels in our experiment were comparable to natural levels. We found no effect of caloric restriction on either total or free baseline CORT, yet food-restricted nestlings begged more intensely than chicks in the high calorie group. Mean plasma concentrations of total and free CORT in experimentally manipulated birds did not differ from levels in free-living nestlings. These results suggest that CORT does not play a role in modulating begging behavior in this species.

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6.2 Introduction

Nest-bound chicks are completely reliant on parental provisioning to acquire nutrients and energy for growth and metabolism. Food deprived nestlings communicate their need for more food by begging. Although begging is generally regarded as an honest signal of nutritional state (Cotton et al. 1996), the mechanisms responsible for ensuring honesty are unclear. Corticosterone (CORT), a steroid hormone that stimulates mobilization of endogenous energy stores and elicits behavioral changes that promote survival (reviewed in Sapolsky et al. 2000), has been proposed as a potential mechanism to ensure honest begging behavior (Kitaysky et al 2001, Loiseau et al. 2008).

In many species, food shortage during growth activates the hypothalamus-pituitary-adrenal (HPA) axis resulting in increased circulating levels of CORT (Nunez-de la Mora et al. 1996, Kitaysky et al. 1999, Walker et al. 2005). Total circulating levels of CORT includes CORT bound to binding globulins (CBGs), as well as an unbound fraction (free CORT) which is thought to represent the concentration of hormone available for diffusion into tissues (Breuner and Orchinik 2002, Love et al. 2004). CBG binds CORT specifically and reversibly, and therefore functions to transport this insoluble hormone to target tissues. CBG may also serve to buffer target tissues from negative effects of elevated CORT and to slow metabolic clearance rates (reviewed in Breuner and Orchinik 2002). In black-legged kittiwakes (*Rissa tridactyla*) and house sparrows (*Passer domesticus*), experimental elevation of CORT increases begging intensity (Kitaysky et al. 2001, Loiseau et al. 2008). For kittiwakes, this increase ultimately resulted in greater provisioning rates by attending adults (Kitaysky et al. 2001). However, chronic elevation of CORT has a variety of negative effects including muscle wasting, impaired cognitive abilities, and reduced immune function (Saino et al. 2003, Kitaysky et al. 2006, Loiseau et al. 2008). Thus, short term elevation of CORT may benefit nestlings by promoting mobilization of stored energy reserves and inducing parents to increase provisioning whereas the costs of chronic elevation may ensure begging behavior is honest (Kitaysky et al. 2001, Loiseau et al. 2008).

However, in species subjected to extended periods of reduced energy intake, the high costs associated with chronic elevation of CORT may lead to non-responsiveness or suppression of the HPA axis during nutritional restriction. Kitaysky et al. (2005) demonstrated that for tufted puffins (*Fratercula cirrhata*), a species in which nestlings routinely experience chronic food shortages, captive chicks respond to severe dietary restriction by decreasing plasma total CORT concentrations. The negative relationship between nutritional state and total CORT presents a potential paradox: total CORT is highest in well-fed puffin chicks and in some species CORT is known to invoke begging behavior. This apparent paradox may be resolved in three manners: 1) CORT may inhibit, rather than promote, begging behavior in tufted puffins, 2) CBGs may be modulated in response to restriction such that free CORT in tufted puffin nestlings is actually negatively correlated with nutritional state, or 3) begging behavior may not be triggered by CORT in this species.

In this study, we experimentally altered the nutritional state of free-living tufted puffin nestlings by preventing adults from provisioning them and feeding them by hand. Chicks responded to hand provisioning by begging, enabling us to examine the relationships between free CORT, total CORT, nutritional state, and begging behavior. We also measured total CORT and free CORT in free-living tufted puffins to determine whether hormone levels of manipulated birds were similar to levels in free-living chicks.

6.3 Methods

We conducted a feeding experiment with tufted puffin nestlings reared in their natural burrows on Cliff Island in Chiniak Bay on Kodiak Island, AK (57°40'N, 152°20W) in 2004. Tufted puffins are monogamous with both parents caring for a single chick raised in a burrow excavated in the soil. We located 13 free-living puffin nestlings during the early stages of chick-rearing and excavated vertical access holes which were patched with flat rocks to permit later access to the nesting chamber. When chicks were estimated to be approximately 10 days old, based on wingchord measurements, we blocked burrow entrances to prevent adults from provisioning their young and began

feeding them one meal per day in the morning. On the first day of the trial, chicks were fed several fish by hand and the remaining fish were placed on the ground inside their burrows. On all subsequent days, nestlings were fed by placing the entire meal on top of a plastic bag and placing the bag on the floor of the nesting chamber. Nestlings were removed and handled (mass taken) prior to feeding every 2-3 days during the first nine days of the trial to ensure they were gaining mass. After day nine, chicks were handled and mass was taken using a spring scale (± 2g) every 4-5 days prior to feeding. We fed nestlings either 120g/day (high calorie treatment; 650 kJ/day, n=6) or 60g/day (low calorie treatment; 325 kJ/day, n=7) of Pacific herring (*Clupea pallasi*) plus a multivitamin supplement. Quantities of fish fed were determined based on a previous captive study (Kitaysky et al. 2005). We expected the caloric intake of the high-calorie group to result in rates of mass growth equivalent to maximum levels observed in the wild and low-calorie birds to grow at slightly below the mean growth rate (reviewed in Piatt and Kitaysky 2002).

We drew blood samples from post-absorptive (24h after last meal) nestlings by puncture of the alar vein with a 24 gauge needle within 3 minutes of handling after 9, 18, and 27 days on the controlled diet. Nestlings were fed and bled between 1000 and 1200. Total CORT values were not affected by time of bleed within 3 minutes of handling (P = 0.33), therefore we consider samples to be indicative of baseline levels (Romero and Reed 2005). On two occasions, we were unable to obtain a baseline sample from one of the nestlings in the high calorie group. We also collected baseline blood samples from free-living puffin nestlings (n=18) captured in their burrows on Chiniak Island (located 22 km east of Cliff Island) in 2005. All free-living nestlings were bled when their wingchord lengths were between 120-130mm to minimize potential effects of developmental stage on CORT. This wingchord length is comparable to that of high-calorie and low-calorie nestlings sampled on day 27 of the feeding trial (low-calorie: 120.3 ± 4.0 mm (SD); high-calorie: 134.5 ± 3.6 mm). Mean mass of free-living nestlings at time of bleeding was 480.0 ± 47.0 g. We sampled only nestlings from burrows that were not connected to other burrows via underground tunnels. Blood was collected in

heparinized 250µl Natelson blood collecting tubes and transferred into 1.5 ml microcentrifuge tubes which were stored on ice for several hours. Blood samples were centrifuged, separated, and plasma samples stored frozen (-70°C) until laboratory analyses were performed.

After 22 and 27 days of provisioning nestlings by hand we recorded whether chicks begged (repeated "peep-peep" vocalization; Piatt and Kitaysky 2002) continuously, intermittently, or not at all during the first twenty seconds following removal from their burrows. We measured begging during handling, rather than when food was added to burrows, to ensure measures of begging were standardized across all chicks. The proximity of excavation holes to the nesting chamber as well as the length and size of burrows varied widely between individuals. Tufted puffins are not known to beg in the absence of parents and we assume begging behavior was in response to hand feeding. Following collection of the final blood sample, we fed all chicks *ad libitum* until they reached the age and size of wild fledglings, at which point we removed obstructions from the burrow entrance and allowed them to fledge on their own initiative or released them to the water.

For each plasma sample, we determined CORT concentration in duplicate following extraction in dichloromethane using direct radioimmunoassay as detailed in Wingfield et al. (1992). We determined recovery values and used these to adjust final assayed concentrations of CORT. Samples from the feeding trial were all run in a single assay and samples from free-living birds were run in a second assay. Mean recovery was 89 ± 3 % (SD) and 94 ± 3 % in the first and second assay, respectively. Intra- and inter-assay variation were 3 % and 4 %, respectively. We determined CBG concentrations as described in Love et al. (2004). We determined optimal incubation time (2 h), final plasma concentration (1:450) and CORT affinity (K_d = 2.33; SE = 0.196). Exogenous CORT was stripped from plasma by incubating each 10 µl sample with 20 µl dextran-coated charcoal solution for 20 min at room temperature prior to centrifugation at 4000 rpm for 10 min at 4°C. We analyzed samples for total binding in triplicate and simultaneously determined non-specific binding in duplicate using unlabeled CORT. We

did not have sufficient plasma to determine CBGs for 5 samples; sample size was between 5-6 for each group in each time period. Following incubation, we separated bound and free radioligand using rapid vacuum filtration (Brandel Harvester) over glass fiber filters (Whatman GF/B, soaked in 25mM Tris with 0.3% PEI for 1 h before filtering). All samples from the experimental feeding trial were run in a single assay and samples from free-living birds were run in a separate assay. Inter-assay variation was 12 % and intra-assay variation ranged between 6-7 %. Free CORT was estimated using the equation of Barsano and Baumann (1989).

We performed all statistical analyses using SAS 9.1 (SAS Institute) and present data as means ± SD. Nestling masses were available for all birds only on days 0, 9, 18, and 27 of the experiment, therefore only these masses were used in statistical analysis. Total and free CORT values were log transformed to meet the assumption of normality. Separate repeated-measures mixed models (PROC MIXED) were used to determine the effects of nutritional regime, age, and their interaction on mass, total CORT, and free CORT. Mixed models permit the inclusion of individuals with missing observations. We tested for differences in begging behavior (intermittent, continuous, or no begging) between low and high calorie groups using a likelihood ratio Chi-square test. We tested for differences in total and free CORT between free-living and experimental nestlings using a Student's t-test. For this analysis, we used only CORT values from day 27 of the experiment (total CORT: $n = 13$; free CORT: $n = 11$), when experimental chicks were at a similar developmental stage as free-living chicks, based on wingchord measurements. Because we found no treatment effects on CORT (see results), we pooled experimental birds for this analysis.

6.4 Results

Body mass did not differ between treatment groups prior to the experiment ($t = -0.37$, $P = 0.72$). Food restriction severely decreased rate of mass gain (Fig. 6.1); mass was significantly affected by age ($F_{3,33} = 601.09$, $P < 0.0001$), treatment ($F_{1,11}=212.77$, $P < 0.0001$), and the interaction between age and treatment ($F_{3,33} = 109.90$, $P < 0.0001$). At

age 37 days, the mean difference in body mass between high and low calorie groups was 227.9g (95% CI: 205.3, 250.2).

Total baseline CORT values were not significantly affected by nutritional restriction ($F_{1,11} = 0.39$, $P = 0.55$) or age ($F_{2,22} = 2.02$, $P = 0.16$; Fig. 6.2a). Similar to total CORT, free CORT was not significantly affected by food restriction ($F_{1,10} = 1.03$, P = 0.33) or age ($F_{2,19} = 0.84$, $P = 0.45$; Fig. 6.2b). Mean values of total CORT and free CORT across treatment groups and ages were 2.6 ± 1.7 and 0.05 ± 0.03 ng/ml, respectively. Total and free baseline CORT in free-living tufted puffin chicks was 3.8 ± 3.7 ng/ml and 0.06 ± 0.08 ng/ml, respectively. These levels were not significantly different from ~37 day old nestlings in the experimental feeding trial (total CORT: $t = 0.09$, $P = 0.9$; free CORT: $t = 0.93$, $P = 0.4$).

Food-restricted nestlings were significantly more likely to beg when removed from their nests compared to chicks on the high calorie diet (22 and 27 days post-restriction: $\chi^2 = 12.20$ and 14.13, $P < 0.001$). At 22 days post restriction, six of the seven food-restricted nestlings begged continuously for >20s when removed from their burrows and one of the seven did not beg. In contrast none of the six chicks from the high calorie group begged when removed from their burrows after 22 days on the controlled diet. After 27 days on the controlled diet, six of the seven food-restricted nestlings begged continuously whereas one of the seven food-restricted nestlings and two of the six nestlings from the high calorie group begged intermittently during the first 20s of handling. The remaining four nestlings from the high calorie group did not make any begging vocalizations when removed from their burrow.

6.5 Discussion

We found that food-restricted nestlings did not have elevated baseline levels of either total CORT or free CORT compared to chicks fed a high calorie diet. Nevertheless, begging behavior appears to be honest in this species with most food-deprived birds begging intensely. These results suggest that in nestlings with an HPA axis that is

uncoupled from nutritional stress, CORT is not responsible for triggering begging behavior.

Effects of CORT on begging behavior appear to be species-specific and may depend on the adrenocortical response to food deprivation. Nutritional restriction causes increases in CORT secretion, which in turn increases begging in kittiwakes (Kitaysky et al. 2001, 2003) and sparrows (Loiseau et al. 2008). In contrast, exogenous CORT had no effect on begging in blue-footed boobie (*Sula nebouxii*) nestlings (Vallarino et al. 2006) even though total CORT levels of chicks in this species rise during food deprivation (Nunez-de la Mora et al. 1996). However, Vallarino et al. (2006) were unable to verify whether CORT implants actually increased plasma CORT concentrations in their study.

The effect of food restriction on HPA activity in tufted puffins apparently depends on the degree of restriction, with moderate restriction having no effect (this study) and severe restriction having suppressive effects (Kitaysky et al. 2005). In our study, levels of total and free CORT in free-living tufted puffins were no different from levels observed in manipulated birds. Baseline total CORT levels of free-living puffin chicks in our study were also comparable to levels in captive-reared chicks, but lower than levels reported for free-living chicks, in the study of Kitaysky et al. (2005). Wild chicks sampled by Kitaysky et al. (2005) were older and it is possible their CORT concentrations were elevated because chicks were approaching fledging age (e.g. Quillfeldt et al. 2007).

An important consideration when manipulating energy intake of growing chicks is determining whether experimental procedures effectively mimic natural conditions. In tufted puffins, chick provisioning and nestling growth rates are highly variable (Piatt and Kitaysky 2002) and nestlings are adapted to adjust metabolic rates to food provisioning (Kitaysky 1999). In our study, mass gain of nestlings during the "linear growth phase" (age 10-30 d; Gjerdrum 2001) in high and low-calorie groups was 17.15 g/day and 6.55 g/day, respectively. Piatt and Kitaysky (2002) review 49 colony years of nestling growth data and report an overall mean growth rate of 10.9 g/day ± 4.7 SD (range: −0.6 to 19 g/day). Thus, growth rates of food-restricted birds in our experiments were less than 1 SD below the overall mean and well within the range reported for wild birds. Growth rates of

the high-calorie group approached the maximum level observed in the wild. However, we only provided chicks with one large meal per day, whereas in the wild they are fed, on average, 2 meals per day from each parent (Piatt and Kitaysky 2002). Kitaysky et al. (2005) found that altering rates of meal delivery had no effect on CORT levels in captive chicks, although effects on begging behavior, if any, are unknown.

In the wild, tufted puffin nestlings make begging vocalizations when adults enter the burrow with food (Piatt and Kitaysky 2002, CTW Pers. Obs.), although variability in begging behavior and its relation to nutritional status has not been studied in free-living puffins. Gjerdrum (2004) found that tufted puffin parents reduced feeding rates in response to supplemental provisioning of their chicks, suggesting they have some means of gauging nestling hunger status. Presumably, tufted puffin parents are able to perceive the nutritional status of their chicks through the intensity of their begging during feeding. Atlantic puffin (*Fratercula arctica*) parents, for example, increased provisioning in response to play-back of recorded begging calls (Harris 1983). Differences in begging behavior were extreme in our study; most nestlings on a high calorie diet made no begging vocalizations whereas all food-restricted chicks did. However, we prevented nestlings from interacting with their parents and therefore our results should be treated with some caution. Further study is required to determine how the changes in behavior measured in our experiment (begging during handling) translate into differences in the solicitation of food from adult puffins.

The high variability in growth rates observed in wild populations suggests that puffin parents are often unable or unwilling to respond to chick begging. Kitaysky et al. (2005) propose that uncoupling HPA activity from nutritional state in puffin chicks is adaptive because it allows slow-growing nestlings to avoid the deleterious effects associated with chronic elevation of CORT. Growing chicks may be particularly susceptible to chronic elevation of CORT as the rate of protein synthesis in skeletal muscle is significantly reduced in the presence of CORT (Klasing et al. 1987) and elevated CORT during development in chicks can impair their ability to associate food distribution with visual cues later in life (Kitaysky et al. 2003, 2006). If long-term

elevation of CORT is costly, then why don't puffin chicks increase CORT in anticipation of a feeding event, as thin-billed prion (*Pachyptila belcheri*) chicks apparently do (Quillfeldt et al. 2007), or in response to the presence of an adult? Although, feeding rates of puffins peak during the morning, chicks are fed throughout the day (Piatt and Kitaysky 2002) and timing of provisioning is likely not predictable enough for an anticipatory increase to be effective. Furthermore, adult puffins often remain in the burrow for less time than the several minutes required to increase circulating levels of CORT following activation of the HPA axis. Thus, CORT levels would increase after the adult has already left the burrow and modulation of the HPA axis in response to adult presence would therefore not be a useful mechanism to increase begging.

It is possible that begging behavior is less costly in species where nestlings routinely encounter prolonged food deprivation. However, this can only be determined once the trigger(s) for begging behavior and the costs associated with these triggers are known. Although CORT is apparently not responsible for modulating begging behavior in tufted puffins, begging is likely regulated by other endocrine systems. The effects of yolk androgen levels on begging behavior is well established in several passerine species (Schwabl 1996, Eising and Groothuis 2003). Additionally, elevated levels of endogenous testosterone are positively correlated with begging intensity in pied flycatchers (*Ficedula hypoleuca*; Goodship and Buchanan 2006) and in thin-billed prions (Quillfeldt et al. 2006), but testosterone implants suppress begging behavior in black-headed gulls (*Larus ridibundus*; Groothuis and Ros 2005). However, total androgen levels were below the minimum detectable level of 0.05ng/ml in free-living and captive >14d post-hatch tufted puffin nestlings sampled in a prior study (Kitaysky unpublished data). Further study is therefore needed to elucidate the hormone(s) responsible for triggering begging behavior in tufted puffins, as well as the mechanism(s) responsible for ensuring this behavior is honest.

6.6 Acknowledgements

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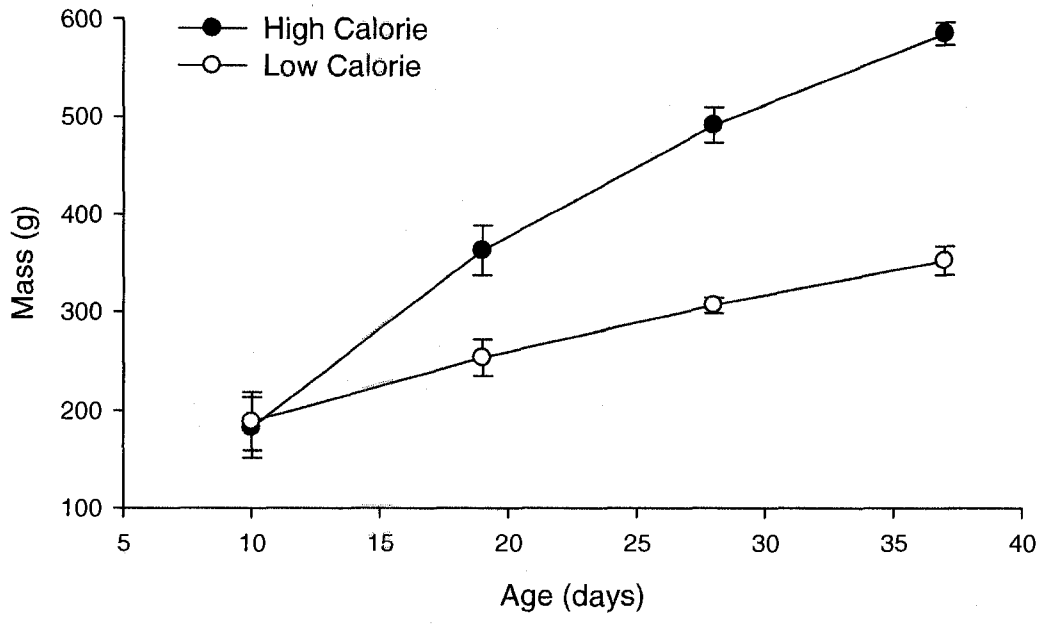

Fig. 6.1 Changes in mass (mean ± SD) in tufted puffins with age for high (650 kJ per day, filled circles, $N = 6$) and low calorie diet (325 kJ per day, open circles, $N = 7$).

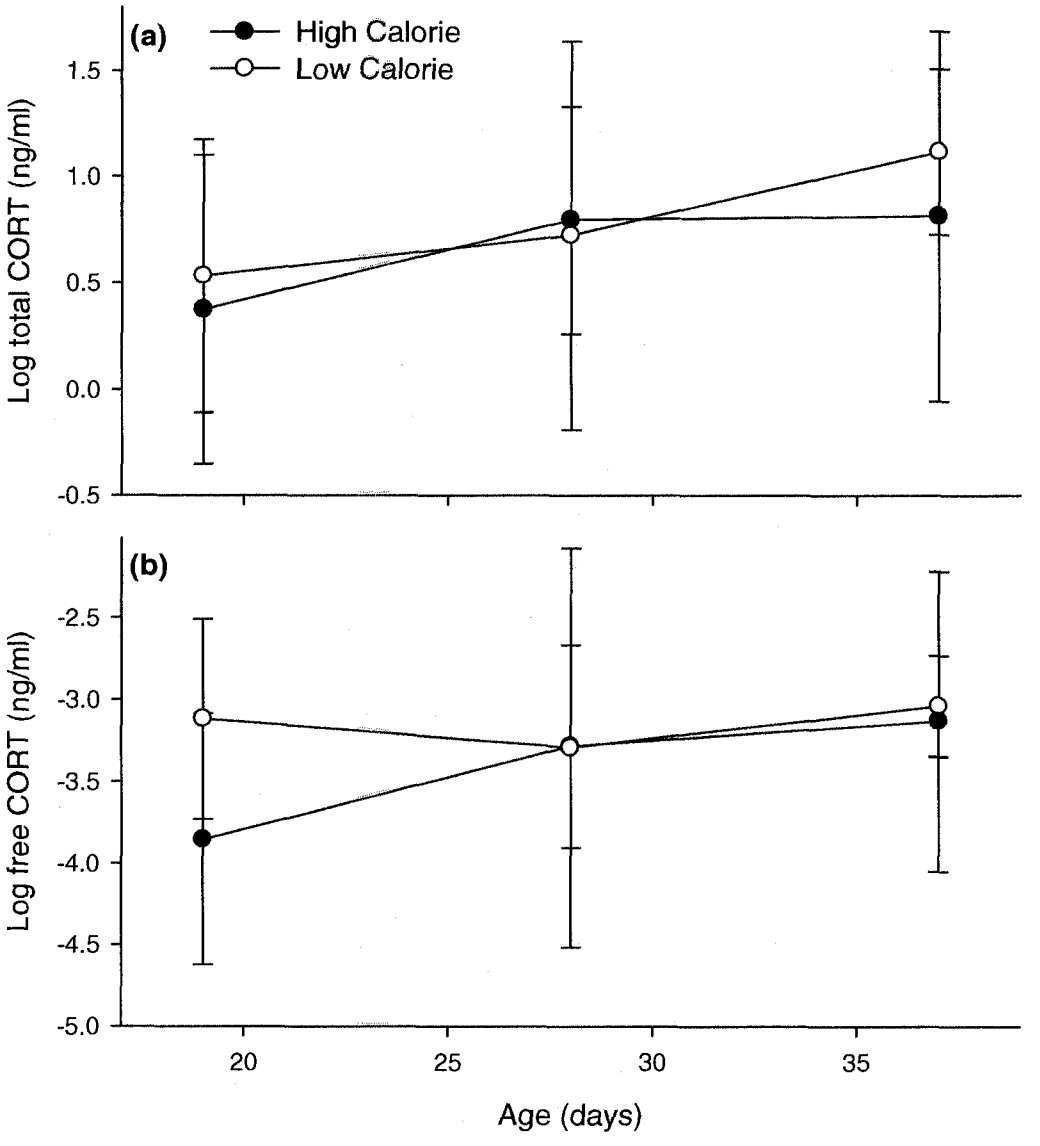

Fig. 6.2 Changes in the values of baseline **a** total CORT and **b** free CORT (mean ± SD) in tufted puffins with age for high (650 kJ per day, filled circles, $N = 5–6$ per time period) and low calorie diet (325 kJ per day, open circles, $N = 5$-7 per time period). Total and free CORT were not significantly affected by age ($P > 0.15$ for both) or treatment ($P > 0.33$ for both).

General Conclusions

Seabirds have long been touted as useful indicators of prey availability, providing valuable information on the spatial and temporal distribution of ecologically important forage fishes (Cairns 1987). Generally, nestling diets are used to provide insight into what prey species are available (e.g. Hatch & Sanger 1992) and measures of reproductive success are used to indicate ecosystem change (e.g. Piatt et al. 2007). However, information on nestling diets is limited in its temporal scope and conventional techniques to measure adult diets are lethal and subject to bias (Votier et al. 2003). The first major goal of this dissertation was to to determine whether information furnished from nestling diets can be extrapolated to adults and to determine whether adult diets depend on stage of breeding. To address this issue, I used molecular techniques (stable isotopes and fatty acid signatures) that permit non-destructive sampling and avoid bias associated with stomach content analysis. However, puffin nestlings routinely experience food restriction during growth and these techniques may be affected by changes in physiological state. Therefore, I first conducted controlled feeding experiments to validate their use.

In Chapter 1, I demonstrated that moderate nutritional restriction during growth affects diet-tissue fractionation factors for nitrogen ($\Delta^{15}N$) and carbon ($\Delta^{13}C$) in tufted puffin nestlings. Contrary to previous studies examining effects of severe chronic food deprivation, I found that blood from food-restricted birds was depleted in ^{15}N compared to well-fed controls. I suggest that these differences stem from more efficient use of nitrogen by food-restricted birds. I also propose that nitrogen diet-tissue fractionation factors are affected by changes in nitrogen-use efficiency associated with growth. I also found that blood $\delta^{13}C$ values were lower in food-restricted birds and suggest this was likely due to incorporation of ^{13}C-depleted lipids into proteinaceous tissues. Although effects of food deprivation were small relative to previously reported effects of trophic level of feeding and foraging location, biologists using stable isotopes need to consider the potential contribution of physiological processes to variability in isotopic fractionation.

In chapter 2, I determined that nutritional restriction during growth also affects adipose tissue fatty acid signatures of tufted puffin nestlings. This result may be a function of decreased rates of fatty acid biosynthesis, selective mobilization or deposition fatty acids into adipose tissue, or effects on fatty acid elongation and/or desaturation pathways. Although food-deprivation had a significant effect on fatty acid signatures, calibration coefficients differed little between well-fed and food-deprived chicks. Furthermore, effects of food restriction on fatty acid signatures were small relative to inter-specific variation in prey fatty acid signatures. More research is needed to determine the consequences of the observed effects on diet estimation using quantitative fatty acid signature analysis (QFASA).

In chapter 3, I used stable isotopes and fatty acid signatures to investigate age and stage-dependent foraging niches of tufted puffins. Based on $\delta^{15}N$ values, I determined that adult tufted puffins increase their trophic level of feeding from the pre-lay period to late chick-rearing by ~0.47-0.68 trophic levels. Fatty acid signatures also indicate diet composition depends on breeding stage. I suggest the effect of reproductive stage on stable isotopes and fatty acid signatures results from stage-dependent foraging strategies of adults, but more work is needed to determine the relative importance of seasonal shifts in prey availability. Analyses of stable isotopes and fatty acid signatures also indicate that chick-rearing adults do not typically feed at a lower trophic level than nestlings, but likely consume a different assortment of prey species. More work is needed to derive species-level estimates of diet composition in breeding adults to better understand the role of puffins in marine food webs.

Breeding seabirds experience a "cost of reproduction" in that current reproductive effort negatively affects the probability of survival as well as reproductive output in subsequent years (Golet et al. 1998, 2004). However, it is currently unclear whether the cost of reproduction is fixed or varies with changes in prey availability. The second major goal of this dissertation was to establish whether breeding is more costly for tufted puffins in years characterized by low rates of nestling growth and survival. To address

this question, I investigated how adult puffins prioritize the competing goals of maintaining their own condition and maximizing the growth rate of their offspring.

In chapter 4, I found that male and female puffins both lost mass during the chick-rearing period in two years characterized by high rates of nestling growth and survival. The body condition index of males declined to a greater degree in the year in which chicks fledged at a lighter weight. In contrast, I did not detect a significant annual difference in the decline of female body condition. More research is needed to determine the proximate and ultimate factors responsible for sex-specific regulation of body condition in tufted puffins and other seabirds.

In chapter 5, I found that baseline corticosterone (CORT) levels during chick-rearing in adult puffins were higher at one colony during two years characterized by low rates of nestling growth and survival compared to a second colony during two years with high rates of growth and survival. Assuming chronic elevation of CORT is costly to survival and/or future reproduction, this supports the premise that the cost of reproduction varies with environmental conditions. I also examined the seasonal dynamics of CORT and corticosteroid binding globulins (CBGs) during reproduction in adult tufted puffins at the colony where nestlings exhibited high rates of growth and survival. I found that plasma concentrations of total CORT, CBG, and free CORT were all much higher during the pre-lay period compared to late incubation and late chick-rearing. Long-term datasets are needed to determine whether the observed seasonal differences are driven by intrinsic or extrinsic factors.

In chapter 6, I determined that tufted puffin nestlings subjected to food restriction increase their begging rates without modulating plasma concentrations of total or free CORT. In contrast, some bird species show increased circulating levels of CORT in response to food-restriction and this increase triggers increased begging behavior (Kitaysky et al. 2001, Loiseau et al. 2008). Our results indicate that hormonal regulation of begging behavior likely differs between species depending on their metabolic response to food restriction. I suggest species that are adapted to chronic food shortages and show pronounced down-modulation of their metabolic rates in response to food restriction will

likely have a hypothalamus-pituitary-adrenal axis that is uncoupled from nutritional state and will not utilize CORT as a trigger to begging behavior.

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